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Assistant Commissioner for Patents
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Presented for filing is a new continuation-in-part patent application of:

Applicant: JUN-ICHI NEZU AND ASUKA OKU

Title: TRANSPORTER GENES

Enclosed are the following papers, including those required to receive a filing date under 37 CFR 1.53(b):

| | <u>Pages</u> |
|----------------------|--------------|
| Specification | 35 |
| Claims | 5 |
| Abstract | 1 |
| Unsigned Declaration | 2 |
| Drawing(s) | 13 |

Enclosures:

— Postcard.

This application is a continuation-in-part (and claims the benefit of priority under 35 USC 120) of PCT/JP98/04009 filed September 7, 1998. The disclosure of the prior applications are considered part of (and are incorporated by reference in) the disclosure of this application.

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Enclosures

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APPLICATION
FOR
UNITED STATES LETTERS PATENT

TITLE: TRANSPORTER GENES
APPLICANT: JUN-ICHI NEZU AND ASUKA OKU

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TRANSPORTER GENES

This application is a continuation-in-part of
PCT/JP98/04009 filed September 7, 1998, and claims priority
from Japanese Application No. 9/260972, filed September 8,
5 1997 and Japanese Application No. 10/156660, filed May 20,
1998.

Field of the Invention

The present invention relates to transporters, proteins
10 involved in transport of substances from the outside to the
inside of cells or vice versa.

Background of the Invention

Recently, the involvement of various transporters
15 localized on the plasma membrane in the uptake system for
nutrients and endogenous substances into cells and their
transport mechanisms have been clarified (Tsuji, A. and
Tamai, I., Pharm. Res., 13, 963-977, 1996). These
transporters recognize the structures of substances to be
20 transported to selectively transport specific substances.
Transporters that recognize the relatively wide range of
structures may possibly recognize foreign substances such
as drugs by mistake, and actively take in them into cells.
It is believed that drugs permeate through the plasma
25 membrane fundamentally by simple diffusion depending on
their physicochemical properties such as molecular size,
hydrophobicity, and hydrogen-binding capacity.
Particularly, in the case of ionic drugs, only molecules in
the non-dissociated form can permeate through the plasma
30 membrane according to the pH partition hypothesis.

However, it has become evident that a number of drugs penetrate through the cell membrane by a specific mechanism other than simple diffusion, that is, an active transport mediated by transporters, in organs that require efficient exchange of intracellular and extracellular substances, including small intestine, uriniferous tubule, placenta, epithelial cells of choroid plexus, hepatocytes, and blood-brain barrier (Tamai, I. and Tsuji, A., *Pharmacia*, 31, 493-497, 1995; Saito, H. and Inui, K., *Igaku no Ayumi*, 179, 393-397, 1996; Tamai, I., *Yakubutsu Dotai* (Pharmacokinetics), 11, 642-650, 1996). For example, it is known that although oral β -lactam antibiotics of the non-esterified type are amphoteric or negatively charged in physiological pHs and sparingly soluble in fat, they are readily absorbed through the intestine. The transport study using the isolated membrane-vesicles system demonstrated that an H^+ -driven peptide transporter localized on the brush-border is involved in the absorption process of these drugs (Tsuji, A. et al., *J. Pharmacol. Exp. Ther.*, 241, 594-601, 1987). Although the specificity of a peptide transport system in terms of the peptide size is so strict as to recognize di- or tri-peptides but not tetrapeptides or larger peptides, it has a rather broad substrate specificity to recognize peptides comprising non-natural amino acids. The peptide transporter mediates transport of β -lactam antibiotics mistakenly due to its broad substrate specificity. This property has been unexpectedly utilized in the clinical field (Tsuji, A., *American Chemical Society* (eds. Taylor, M. D., Amidon, G. L.), Washington, D. C., 101-134, 1995). Furthermore, a possibility that a transporter is also involved in permeation of substances

with a high hydrophobicity such as fatty acids through the plasma membrane has been reported (Schaffer, J. and Lodish, H., Cell, 79, 427-436, 1994).

Since various transporters are supposed to be distributed in organs and cells based on the physiological roles of the organs and cells, their distribution and functions may be specific to organs. Therefore, transporters are expected to be used to impart an organ-specificity to pharmacokinetics. In other words, an organ-specific drug delivery system (DDS) can be constructed utilizing transporters. If drug absorption solely relied on simple diffusion is improved by elevating its hydrophobicity, an effect of the drug obtained in the initial transport in the liver can be enhanced and the drug can non-specifically translocates into any organ. In addition, it would also be possible to increase the drug absorption independently of its fat-solubility by designing the drug utilizing the substrate specificity of transporters (Hayashi, K. et al., Drug Delivery System, 11, 205-213, 1996). For this purpose, it is necessary to identify various transporters at the molecular level and analyze their properties in detail. However, their molecular level identification are greatly behind studies on their membrane physiology because they are difficult to handle biochemically and require complicated processes in their functional assays.

Recently, cDNAs of several transporters have been cloned by the expression cloning method using *Xenopus* oocytes, a foreign gene expression system, and structural homology among them has been revealed (Fei, Y.-J. et al., Nature, 368, 563-566, 1994). For example, Koepsell et al. cloned

an organic cation transporter, OCT1, which is assumed to be localized on a basement membrane, using the expression cloning method in 1994 (Grundemann, D. et al., Nature, 372, 549-552, 1994). Subsequently, OCT2 was identified by
5 homology cloning based on the sequence of OCT1 (Okuda, M. et al., Biochem. Biophys. Res. Commun., 224, 500-507, 1996). OCT1 and OCT2 show homology as high as 67% to each other (Grundemann, D. et al., J. Biol. Chem., 272, 10408-10413, 1997). Both of them are intensely expressed in the
10 kidney, but differ in the organ distribution; OCT1 is also expressed in the liver, colon, and small intestine, while OCT2 expression is specific to the kidney.

Only a few reports on identification of transporters at the molecular level, including the reports, are available,
15 and there would be many unknown transporters that may be clinically useful.

Summary of the Invention

An object of this invention is to provide a family of
20 novel transporter genes, proteins encoded by these genes, and their use.

The present inventors have screened a fetal gene library constructed using the subtractive method by random sequencing based on a working hypothesis that fetal genes
25 include those which are involved in various disorders including cancer and are specifically or intensely expressed in fetal tissues. The inventors discovered an unknown gene showing a significant homology with those for organic cation transporters, OCT1 and OCT2, and attempted
30 to isolate this gene, which was assumed to encode a novel

transporter. Thus, the inventors succeeded in isolating the desired gene by screening a cDNA library derived from human fetus. Furthermore, the inventors studied the transporter activity of a protein encoded by the isolated human gene and found that the protein, in fact, functioned as a transporter for various organic cations. The inventors also succeeded in isolating a mouse gene corresponding to the isolated human gene.

This invention relates to a family of novel transporter genes, proteins encoded by these genes, and their use, and more specifically to:

(1) a protein comprising an amino acid sequence set forth in SEQ ID NOs: 1, 3, 22, or 27, or a protein comprising said amino acid sequence in which one or more amino acid residues are substituted, deleted, or added, and having an activity to transport an organic cation;

(2) a protein encoded by a DNA hybridizing to a DNA comprising nucleotide sequence according to SEQ ID NOs: 2, 4, 23, or 28, and having an activity to transport an organic cation;

(3) a DNA encoding the protein according to (1) or (2);

(4) a vector comprising the DNA according to (3);

(5) a transformant expressibly carrying the DNA according to (3);

(6) a method for producing the protein according to (1) or (2), the method comprising culturing the transformant according to (5);

(7) an antibody that binds to the protein according to (1) or (2); and

(8) a DNA specifically hybridizing to a DNA comprising a nucleotide sequence set forth in SEQ ID NOs: 2, 4, 23, or 28, and consisting of at least 15 nucleotides.

Nucleotide sequences of cDNAs of novel human transporters isolated by the present inventors are shown in SEQ ID NO: 2 (designated as "human OCTN1") and SEQ ID NO: 4 (designated as "human OCTN2"), respectively. Amino acid sequences of proteins encoded by these cDNAs are shown in SEQ ID NO: 1 and SEQ ID NO: 3, respectively. Amino acid sequences of these two proteins included in the transporter proteins of this invention showed such a high overall homology as about 76%, and both of them retained the following consensus sequence which is conserved in various types of transporters including the glucose transporter: [Leu, Ile, Val, Met, Ser, Thr, Ala, Gly]-[Leu, Ile, Val, Met, Phe, Ser, Ala, Gly]-Xaa<2>-[Leu, Ile, Val, Met, Ser, Ala]-[Asp, Glu]-Xaa-[Leu, Ile, Val, Met, Phe, Tyr, Trp, Ala]-Gly-Arg-[Arg, Lys]-Xaa<4-6>-[Gly, Ser, Thr, Ala] (Maiden, M. C. et al., Nature, 325, 641-643, 1987). In fact, these proteins have an activity to transport various organic cations (see Examples 6 to 8).

The present inventors also isolated mouse genes corresponding to the above-described human OCTN1 and human OCTN2. Nucleotide sequences of the isolated cDNAs are shown in SEQ ID NO: 23 (designated as "mouse OCTN1") and SEQ ID NO: 28 (designated as "mouse OCTN2"), respectively. Amino acid sequences of proteins encoded by these cDNAs are shown in SEQ ID NOs: 22 and 27, respectively.

Transporter proteins of this invention also include those having the additional activity to transport substances other than organic cations as far as they retain the organic cation transport activity. Organic cations include, for example, TEA, carnitine, quinidine, and pyrilamine, but are not limited to them. They also include carcinostatic agents such as actinomycin D, etoposide, vinblastine, daunomycin, etc. Transporter proteins of this invention include those having the activity to transport organic cations not only from the outside to the inside of cells but also from the inside to the outside of cells.

Transporter proteins of this invention can be prepared as recombinant proteins using recombination techniques or natural proteins. Recombinant proteins can be prepared, for example, as described below, by culturing cells transformed with DNA encoding proteins of this invention. Natural proteins can be isolated from the kidney and cancer cell strains such as Hela S3, which highly express the proteins of this invention, by the method well known to those skilled in the art, for example, affinity chromatography using an antibody of this invention described below. The antibody may be either polyclonal or monoclonal. A polyclonal antibody can be prepared by purifying serum obtained from, for example, a small animal such as a rabbit immunized with proteins of this invention by known methods, for example, ammonium sulfate precipitation, protein A or protein G column, DEAE-ion exchange column chromatography, affinity column chromatography coupled with the protein of this invention, etc. A monoclonal antibody can be prepared by immunizing a small animal such as a mouse with the protein of this

invention, excising the spleen from the mouse, grinding the tissue into cells, fusing them with mouse myeloma cells using a fusing agent such as polyethylene glycol, and selecting a clone that produces an antibody to proteins of this invention out of fused cells (hybridomas) thus produced. Then, hybridomas thus selected are transplanted into the abdominal cavity of a mouse, and the ascites is collected from the mouse. A monoclonal antibody thus obtained can be purified by, for example, ammonium sulfate precipitation, protein A or protein G column, DEAE-ion exchange column chromatography, affinity column chromatography coupled with the protein of this invention, etc. When the antibody thus obtained is administered to human subjects, a humanized antibody or a human antibody is advantageously used to reduce the immunogenicity. An antibody can be humanized by, for example, the CDR grafting method comprising cloning an antibody gene from monoclonal antibody-producing cells and grafting the epitope portion thereof into an existing human antibody. A human antibody can be prepared by the usual method for preparing a monoclonal antibody except for immunizing a mouse whose immune system is replaced with the human's.

The term "substantially pure" as used herein in reference to a given polypeptide means that the polypeptide is substantially free from other biological macromolecules. The substantially pure polypeptide is at least 75% (e.g., at least 80, 85, 95, or 99%) pure by dry weight. Purity can be measured by any appropriate standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A "conservative amino acid substitution" is one in which an amino acid residue is replaced with another residue having a chemically similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

It is also possible for those skilled in the art to prepare proteins having functions equivalent to the transporter proteins of this invention (human OCTN1, human OCTN2, mouse OCTN1, and mouse OCTN2) by appropriately modifying amino acid residues of the proteins by, for example, substitution, using well known methods. Mutation of amino acids of the proteins can occur also spontaneously. Such mutant proteins which are obtained by altering the amino acid sequence of the transporter proteins of this invention by substitution, deletion, or addition of amino acid residues, and are functionally equivalent to those of the transporter proteins are also included in the proteins of this invention. Herein, "functionally equivalent" means that proteins have an activity to transport organic cations. Methods well known to those skilled in the art for altering amino acids include, for example, the site-specific mutagenesis system

by PCR (GIBCO-BRL, Gaithersburg, Maryland), site-specific mutagenesis by oligonucleotide (Kramer, W. and Fritz, H. J. (1987) Methods in Enzymol., 154: 350-367), Kunkel's method (Methods Enzymol., 85, 2763-2766 (1988)), etc. The number of amino acids that can be substituted is usually 10 amino acid residues or less, preferably 6 or less, and more preferably 3 or less. The site of substitution, deletion, or addition of amino acid residues is not particularly limited as far as the activity of proteins of this invention is retained. It is possible to detect the transporter activity of proteins, for example, by the method described below in Example 6.

It is routine for those skilled in the art to obtain proteins functionally equivalent to the transporter proteins of this invention by isolating and using DNAs highly homologous to the DNA sequences encoding the transporter proteins of this invention (human OCTN1, human OCTN2, mouse OCTN1, and mouse OCTN2) or portions thereof using hybridization techniques (Sambrook, J. et al., Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. press, 1989), etc. These proteins functionally equivalent to those of transporter proteins of this invention are also included in proteins of this invention. Here, "functionally equivalent" means that proteins have an activity to transport organic cations. DNAs that hybridize to the DNAs encoding the proteins of this invention can be isolated from other organisms, for example, rats, rabbits, cattle, etc. as well as humans and mice. Especially, tissues such as the kidney are suitable as sources of such DNAs. These DNAs isolated using hybridization techniques usually have a high homology with the above-described DNAs

encoding the transporter proteins of this invention. "High
homology" means at least 70% or more, preferably at least
80% or more, and more preferably at least 90% or more of
amino acid sequence identity. The "percent identity" of
5 two amino acid sequences or of two nucleic acids is
determined using the algorithm of Karlin and Altschul
(Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990), modified
as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA
90:5873-5877, 1993). Such an algorithm is incorporated
10 into the NBLAST and XBLAST programs of Altschul et al. (J.
Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches
are performed with the NBLAST program, score = 100,
wordlength = 12. BLAST protein searches are performed with
the XBLAST program, score = 50, wordlength = 3. Where gaps
15 exist between two sequences, Gapped BLAST is utilized as
described in Altschul et al. (Nucleic Acids Res. 25:3389-
3402, 1997). When utilizing BLAST and Gapped BLAST
programs, the default parameters of the respective programs
(e.g., XBLAST and NBLAST) are used. See
20 <http://www.ncbi.nlm.nih.gov>.

One example of hybridization conditions for isolating
such DNAs is as follows. That is, after the pre-
hybridization at 55°C for 30 min or more in the "ExpressHyb
Hybridization Solution" (CLONTECH), a labeled probe is
25 added, and hybridization is performed by heating the
reaction mixture at 3°C to 55°C for 1 h or more. Then, the
reaction product is successively washed in 2 x SSC and 0.1%
SDS three times at room temperature for 20 min, and then in
1 x SSC and 0.1% SDS once at 37°C for 20 min. More
30 preferable conditions are as follows. After the pre-
hybridization at 60°C for 30 min or more in the "ExpressHyb

Hybridization Solution" (CLONTECH), a labeled probe is added, and hybridization is performed by heating the reaction mixture at 60°C for 1 h or more. Then, the reaction product is successively washed in 2 x SSC and 0.1% SDS three times at room temperature for 20 min, and then in 1 x SSC and 0.1% SDS twice at 50°C for 20 min. Still more preferable conditions are as follows. After pre-hybridization at 68°C for 30 min or more in the "ExpressHyb Hybridization Solution" (CLONTECH), a labeled probe is added, and hybridization is performed by heating the reaction mixture at 68°C for 1 h or more. Then, the reaction product is successively washed in 2 x SSC and 0.1% SDS three times at room temperature for 20 min, and then in 0.1 x SSC and 0.1% SDS twice at 50°C for 20 min.

The present invention also relates to DNAs encoding the above-described transporter proteins of this invention. DNAs of this invention may be cDNA, genomic DNAs, and synthetic DNAs. The DNAs of the present invention can be used for producing proteins of this invention as recombinant proteins. That is, it is possible to prepare proteins of this invention as recombinant proteins by inserting DNAs encoding proteins of this invention (e.g. DNAs comprising the nucleotide sequences set forth in SEQ ID NOs: 2, 4, 23, and 28) into an appropriate expression vector, culturing transformants obtained by transfecting suitable cells with the vector, and purifying the proteins thus expressed. Cells to be used for producing recombinant proteins include, for example, mammalian cells such as COS cells, CHO cells, NIH3T3 cells, etc., insect cells such as Sf9 cells, yeast cells, *E. coli*, and so on. Vectors used for the intracellular expression of recombinant proteins

vary depending on host cells, including, for example, pcDNA3 (Invitrogen), pEF-BOS (Nucleic Acids Res., 1990, 18(7), p5322), etc. for mammalian cells, "BAC-to-BAC baculovirus expression system" (GIBCO BRL), etc. for insect cells, "Pichia Expression Kit" (Invitrogen), etc. for yeast cells, pGEX-5X-1 (Pharmacia), "QIAexpress system" (Qiagen), etc. for *E. coli*. Host cells can be transformed with vectors, for example, by the calcium phosphate method, the DEAE-dextran method, the method using cationic liposome DOTAP (Boehringer Mannheim), the electroporation method, the calcium chloride method, etc. Recombinant proteins can be purified from recombinants thus obtained using standard methods, for example, as described in "The Qiaexpressionist Handbook, Qiagen, Hilden, Germany."

The present invention also relates to DNAs consisting of at least 15 nucleotides that specifically hybridize to the DNAs encoding proteins of this invention. Herein, "specifically hybridize" means that a DNA does not cross-hybridize to other DNAs encoding other proteins under usual hybridization conditions, preferably under the stringent hybridization conditions. Such a DNA can be utilized as a probe for detecting and isolating DNA encoding the protein of this invention, and as a primer for amplifying the DNA.

By hybridization under "stringent conditions" is meant hybridization at 37°C, 1 X SSC, followed by washing at 42°C, 0.5 X SSC.

An "isolated nucleic acid" is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes. The term therefore covers, for

example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it

5 naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, 10 a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are nucleic acids present in mixtures of 15 different (i) DNA molecules, (ii) transfected cells, or (iii) cell clones: e.g., as these occur in a DNA library such as a cDNA or genomic DNA library.

The transporter proteins of this invention can be used to control internal absorption and dynamics of drugs.

20 Based on the results of detailed analysis of the substrate specificity of transporter proteins of this invention, drugs can be designed so as to be transported by these transporters and absorbability of the drugs mediated by these transporter proteins can be improved. Conventional 25 modifications to enhance hydrophobicity are no longer necessary for drugs so designed, which enables speedily and efficiently developing water-soluble drugs that are easy to handle. The drugs thus developed is thought to be absorbed principally depending on the internal distribution pattern 30 of transporter proteins of this invention, and an organ-specific delivery of the drugs thus becomes possible.

Especially, if the transporter proteins of this invention are distributed in the target organ of a drug, an ideal drug delivery system (DDS) can be developed. If a drug is to be absorbed mediated by not the transporter proteins of this invention but other transporters, the drug can be designed so as to be specific to other transporter proteins by designing it considering the substrate specificity of the transporter proteins of this invention. Since the transporter proteins of this invention are present in the kidney, it is possible to reduce the nephrotoxicity produced by a drug by designing the drug so that it can be readily excreted by the transporter proteins of this invention.

Another possible application of this invention is to develop a drug targeting the transporter proteins of this invention. The transporters play important roles in the absorption mechanism of nutrients and drugs, or the excretion mechanism of drugs and internal metabolites. Thus, damage or abnormal elevation of the transporter's functions may cause some disorders. It is considered to be efficacious against such disorders to administer a drug containing a compound that inhibits or enhances functions of the transporter proteins of this invention, or regulates the expression level of the transporter gene of this invention and the amount of the transporter proteins. The DNAs of this invention can be used in gene therapy for disorders caused by abnormalities in the activity and expression of the proteins of this invention. In this case, the DNA of this invention are inserted to an adenovirus vector (e.g. pAdexLcw), a retrovirus vector (e.g. pZIPneo), etc., and administered into the body by

either *ex vivo* method or *in vivo* method. Gene therapy can also be performed by administering a synthetic antisense DNA to the body either directly or after inserted into the above-described vector.

5 Especially, since "OCTN2" included in the transporter proteins of this invention efficiently transports carnitine, chemotherapy with compounds to control the activity of "OCTN2" or gene therapy using the "OCTN2" gene is considered to be efficacious against various
10 pathological conditions such as fatty liver, myocardiopathy, myopathy, etc. caused by hypocarnitinemia.

The transporter proteins of this invention are expressed in a variety of cancer cell strains, which suggests that the proteins may transport drugs into tumor cells. If this
15 is the case, it is possible to develop carcinostatics that will be readily absorbed mediated by the transporter proteins of this invention. On the contrary, mechanisms to transport and excrete substances by the transporter proteins of this invention may function to excrete
20 carcinostatics in tumor cells so that the cells acquire resistance to drugs. If the transporter proteins of this invention are involved in a mechanism of tumor cells to acquire drug resistance, a carcinostatic effect can be enhanced by a combined use of inhibitors of the transporter
25 proteins of this invention with carcinostatics.

Brief Description of the Drawings

Fig. 1 represents hydrophobicity plots of human OCTN1 and human OCTN2 according to Kyte & Doolittle's calculating
30 formula with a window of nine amino acid residues.

Numerals on the plots indicate putative transmembrane regions.

Fig. 2 represents electrophoretic patterns showing the results of Northern blot analysis of human OCTN1.

5 Fig. 3 compares the amino acid sequence of human OCTN1 with that of human OCTN2. Amino acid residues conserved in both transporters are shaded. Sequences coinciding with the consensus sequences of sugar transporter and the ATP/GTP binding site are indicated by "+" and "*",
10 respectively.

Fig. 4 represents electrophoretic patterns showing the results of Northern blot analysis of human OCTN2.

Fig. 5 is a graph showing the TEA-absorbing activity of human OCTN1. Clear circles represent untreated cells, and
15 solid circles represent human OCTN1-transfected cells.

Fig. 6 is a graph showing effects of the cold TEA added in the experimental system in Fig. 5. In this graph, solid circles represent human OCTN1-transfected cells, and clear circles represent cells containing the vector with no
20 insert. Clear triangles indicate the net uptake induced by human OCTN1 obtained by subtracting the clear circle values from the corresponding solid circle values.

Fig. 7 is a graph showing TEA concentration-dependency of the TEA-absorbing activity of human OCTN1.

25 Fig. 8 is a bar graph showing the activity of the human OCTN1-transfected cells to absorb substances other than TEA.

Fig. 9 is a bar graph showing the results of transport experiments using *Xenopus* oocytes. Bars indicated with

"OCTN1" and "Water" represent the uptake activity of the human OCTN1-injected cRNA oocytes and that of the water-injected oocytes (containing no cRNA), respectively.

Uptakes of TEA, carnitine, mepyramine, quinidine, and actinomycin D were observed in human OCTN1 cRNA-injected oocytes, whereas water-injected oocytes (containing no cRNA) exhibited almost no uptake activity.

Fig. 10 is a bar graph showing the results of transport experiments for carcinostatics in *Xenopus* oocytes. Bars indicated with "OCTN1" and "Water" represent the uptake activity of the human OCTN1 cRNA-injected oocytes and that of the water-injected oocytes (containing no cRNA), respectively. Uptakes of actinomycin D, etoposide, vinblastine, and daunomycin were observed in the human OCTN1 cRNA-injected oocytes.

Fig. 11 is a bar graph showing the results of transport experiments with human OCTN1 and human OCTN2 in HEK293 cells. Human OCTN1 has the efficient transport activity for TEA and human OCTN2 for carnitine.

Fig. 12 is a graph showing the results of Na⁺-dependency of the carnitine transport activity of human OCTN2. Human OCTN2 exhibits a time-dependent carnitine transport activity (clear circle) in the presence of Na⁺, while no such activity in the absence of Na⁺ (solid circle), indicating that the carnitine transport activity of human OCTN2 depends on the presence of Na⁺.

Fig. 13 shows the expression of mouse OCTN1 and mouse OCTN2 genes detected by RT-PCR amplification in each tissue. G3PDH serves as a control, indicating that the amount of cDNA in each tissue is uniform.

Detailed Description

The present invention is described below in more detail with reference to examples, but is not construed being
5 limited thereto.

Example 1 Construction of a subtraction library

A subtraction library was constructed using the PCR-Select™ cDNA Subtraction Kit (CLONTECH) principally
10 according to the method of Luda Diatchenko (Diatchenko, L. et al., Proc. Natl. Acad. Sci. USA, 93, 6025-6030, 1996).

First, double-stranded cDNAs were synthesized from poly(A)⁺ RNAs derived from human fetal liver and adult liver by the standard method using MMLV reverse transcriptase.
15 These cDNAs were blunt-ended with T4 DNA polymerase and cleaved with RsaI. A part of the cDNAs derived from fetal liver (tester) was divided in two portions, and they were separately ligated to two different adapters, adapter 1 and adapter 2, respectively (Table 1). A 120-fold excess of
20 cDNA derived from adult liver (driver) was added to each of the above-described tester samples. The mixture was heat-denatured and subjected to the primary hybridization at 68°C for 8 h. After these two reaction mixtures from the primary hybridization were mixed together without heat-
25 denaturation, an excessive amount of the heat-denatured driver was further added thereto, and the mixture was subjected to the secondary hybridization at 68°C for about 16 h. The resulting reaction solution was diluted with a dilution buffer and incubated at 75°C for 7 min. After the
30 shorter strands of adapters were removed, the reaction

solution was used as a template for PCR. PCR using primers
1 (5'-CTAATACGACTCACTATAGGGC-3', SEQ ID NO: 5) and
2 (5'-TGTAGCGTGAAGACGACAGAA-3', SEQ ID NO: 6) corresponding
to the adapters selectively amplified only cDNAs having
5 different adapters at their both ends (subtracted
cDNAs) (suppression PCR). PCR was carried out using a
portion of the resulting cDNA as a template, and nested PCR
primers 1 (5'-TCGAGCGGCCGCCCCGGGCAGGT-3', SEQ ID NO: 7) and
2 (5'-AGGGCGTGGTGCGGAGGGCGGT-3', SEQ ID NO: 8), which are
10 further inwardly located from the PCR primers 1 and 2, to
obtain products with further elevated selectivity. PCR
products thus obtained were purified using the QIAquick PCR
Purification kit (QIAGEN), and cloned into the pT7Blue-T
vector (Novagen) by the TA cloning method to construct a
15 subtraction library.

Table 1

| | |
|--------------|---|
| Adapter 1 | 5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCCGGGCAGGT-3' 3'-GGCCCGTCCA-5' |
| Adapter 2 | 5'-TGTAGCGTGAAGACGACAGAAAGGGCGTGGTGCGGAGGGCGGT-3' 3'-GCCTCCCGCCA-5' |

20 The longer strand of the partially single stranded DNA of
Adapters 1 and 2 are designated SEQ ID NOs:29 and 30,
respectively, and the shorter strand of Adapters 1 and 2
are designated SEQ ID NOs:31 and 32, respectively.

Example 2 cDNA cloning

To analyze fetal genes, the subtraction library derived from the fetal liver was screened by random sequencing.

Homology search (Blastx) of Expressed Sequence Tags (ESTs)

5 thus obtained found a clone, OCTN1 (fls 631) (292 bp) encoding amino acid sequence having significant homology with the known organic cation transporters, OCT1 (Grundemann, D. et al., Nature, 372, 549-552, 1994) and OCT2 (Okuda, M. et al., Biochem. Biophys. Res. Commun., 10 224, 500-507, 1996). Since the sequence of this clone was novel and assumed to be a fragment derived from a new transporter gene, cDNA comprising the whole open reading frame (ORF) of this gene was cloned.

The human fetal liver 5'-stretch cDNA library (CLONTECH) 15 was screened using the original OCTN1 clone obtained from the subtraction library derived from fetal liver as a probe. An insert of the original OCTN1 clone was amplified by PCR using M13 P4-22 and M13 P5-22, and labeled with

[α -³²P]dCTP by the random primer method using the Ready-to 20 Go DNA labeling beads (Pharmacia) to serve as a probe. Hybridization was carried out at 68°C in the ExpressHyb Hybridization Solution (CLONTECH) according to the method recommended by the manufacturer. Final washing was performed at 50°C in 0.1 x SSC and 0.1% SDS. Screening 25 about 5 x 10⁵ phage clones finally isolated seven positive clones. cDNA inserts of these clones were amplified by PCR using vector primers designed based on a sequence of the λ gt10 vector (GT10 S1 5'-CTTTTGAGCAAGTTCAGCCT-3', SEQ ID NO: 9, and GT10 A1 5'-AGAGGTGGCTTATGAGTATTTCTT-3', SEQ ID 30 NO: 10), or primers designed based on the decoded cDNA sequences. The PCR products thus obtained were directly

sequenced to determine the nucleotide sequences. Some regions that were difficult to be amplified were subjected to PCR using 7-deaza dGTP as a substrate base (McConlogue, L. et al., *Nucleic Acids Res.*, 16, 9869, 1988).

5 Sequencing of cDNA inserts of these clones revealed that the human OCTN1 gene contains an ORF encoding a protein consisting of 551 amino acid residues (putative molecular weight of about 62,000). Data base search using this whole amino acid sequence confirmed that it has a significant
10 overall homology (about 34%) with OCT1 and OCT2. Hydrophobicity profile of this sequence obtained by Kyte & Doolittle's calculating formula (Kyte, J. and Doolittle, R. F., *J. Mol. Biol.*, 157, 105-132, 1982) very closely resembled those of OCT1 and OCT2, indicating that the
15 sequence has eleven to twelve putative transmembrane hydrophobic regions (Fig. 1). This sequence contained one consensus sequence of sugar transporter, ([Leu, Ile, Val, Met, Ser, Thr, Ala, Gly]-[Leu, Ile, Val, Met, Phe, Ser, Ala, Gly]-Xaa<2>-[Leu, Ile, Val, Met, Ser, Ala]-[Asp, Glu]-
20 Xaa-[Leu, Ile, Val, Met, Phe, Tyr, Trp, Ala]-Gly-Arg-[Arg, Lys]-Xaa<4-6>-[Gly, Ser, Thr, Ala]), (160 to 175). This consensus sequence is present in the glucose transporters GLUT1 to GLUT7 in mammalian cells, and also present in
25 transporters (Maiden, M. C. et al., *Nature*, 325, 641-643, 1987). Furthermore, putative N-linked glycosylation sequences (N-X-[ST]) were found in the amino acid sequence of human OCTN1 at four sites (57 to 59, 64 to 66, 91 to 93, and 304 to 306), and also five putative protein kinase C
30 phosphorylation sites ([ST]-X-[RK]) (164 to 166, 225 to 227, 280 to 282, 286 to 288, and 530 to 532). In addition,

the consensus sequence ([Ala, Gly]-Xaa(4)-Gly-Lys-[Ser, Thr]) of the ATP/GTP binding site is also found. This consensus sequence of the ATP/GTP binding site is also present in the ATP binding protein or GTP binding protein, such as kinases and ras family proteins, and that ATP or GTP binds to this site (Walker, J. E. et al., EMBO J., 1, 945-951, 1982). This sequence is present in the so-called ATP Binding Cassette (ABC) type transporter, and involved in the substance transport using the energy generated by hydrolysis of ATP (Higgins, C. F. et al., J. Bioenerg. Biomembr., 22, 571-592, 1990; Urbatsch, I. L. et al., J. Biol. Chem., 270, 26956-26961, 1995). Presence of this consensus sequence indicates that OCTN1 protein may be an ATP or GTP-dependent transporter.

Nucleotide sequencing was performed by the cycle-sequencing method with a plasmid DNA prepared by the alkaline-SDS method or a PCR product obtained by colony PCR, etc. as a template using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit With AmplyTaq DNA Polymerase, FS, followed by decoding with the ABI 377 DNA Sequencer (Perkin Elmer). Colony PCR was carried out by directly suspending a colony of a recombinant in a PCR reaction solution containing vector primers M13 P4-22 (5'-CCAGGGTTTCCCAGTCACGAC-3', SEQ ID NO: 11) and M13 P5-22 (5'-TCACACAGGAAACAGCTATGAC-3', SEQ ID NO: 12). After the completion of PCR, a DNA insert thus amplified was separated from unreacted primers and nucleotides by gel filtration, etc. to serve as a template for sequencing.

Example 3 Northern analysis

Distribution of human OCTN1 in tissues was investigated by Northern analysis (Fig. 2). A 3'-end fragment of human OCTN1 (the latter half from around the base 1,100) was

5 labelled with [α -³²P]dCTP by the random primer method using the Ready-to Go DNA labeling beads (Pharmacia) to serve as a probe. Hybridization was performed using the Multiple Tissue Northern (MTN) Blot - Human, Human III, Human IV, Human Fetal II, and Human Cell lines (CLONTECH) at 68°C in
10 the ExpressHyb Hybridization Solution (CLONTECH) according to the method recommended by the manufacturer. Final washing was performed at 50°C in 0.1 x SSC and 0.1% SDS.

As a result, RNA of about 2.5 kb was strongly expressed in the fetal liver and adult-derived tissues such as the
15 kidney, bone marrow, and trachea. Besides those tissues, the RNA band was also weakly detected in the fetal kidney and lung, and adult tissues including skeletal muscle, lung, placenta, prostate, spleen, and spinal cord. The RNA expression was also detected in tumor cell lines such as
20 HeLa S3, K562, SW480, and A549, and especially, its very intense expression was observed in HeLa S3.

Example 4 Cloning of human OCTN2 cDNA

Data base search using the entire nucleotide sequence of
25 "human OCTN1" can detect very similar sequences thereto in several parts of the nucleotide sequence of P1 phage clones (P1 H24 clones, GenBank accession No. L43407, L43408, L46907, L81773, and L43409) derived from q regions of human chromosome 5. The parts having similarity with the
30 nucleotide sequence of human OCTN1 are separated by the

sequences having no similarity to the human OCTN1 sequence. The sequence obtained by connecting these similar parts with each other with reference to the sequence of human OCTN1 has a high homology over a wide range with human OCTN1, indicating the presence of OCTN1 homologues. The genomic sequence registered in data base was an incomplete one without covering the entire coding region, and, from only this sequence, it was impossible to know the complete structure of a protein partially encoded by the sequence.

Therefore, cDNA cloning of this OCTN1 homologous gene (OCTN2) was performed to determine the coded protein structure. First, 631R S4 primer (5'-GTGCTGTTGGGCTCCTTCATTTCA-3', SEQ ID NO: 13) and 631R A1 primer (5'-AGCTGCATGAAGAGAAGGACACTG-3', SEQ ID NO: 14) were prepared based on sequences of these P1 phage clones. PCR was performed using a set of these primers and cDNA synthesized from poly(A)⁺ RNA derived from the human adult kidney (CLONTECH) as a template, under the following conditions: 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min,; and 1 cycle of 72°C for 10 min, resulting in amplification of about 900 bp fragment. This fragment was subcloned into the pT7Blue-T vector (Novagen) by the TA cloning method to determine its nucleotide sequence, which clearly showed a very high overall homology with human OCTN1. Therefore, this gene was designated as human OCTN2, and longer cDNAs were cloned.

The cDNA library derived from the human kidney was screened using the cDNA insert of this clone as a probe in the same manner as for human OCTN1 cDNA cloning, and cDNA containing the entire coding region of human OCTN2 was

cloned by a procedure for isolating longer clone and the Rapid Amplification of cDNA Ends (RACE) method (Chenchik, A., Moqadam, F., and Siebert, P. (1995), CLONTECHniques X, 5-8), etc. to determine its structure (SEQ ID NO: 4).

5 Specifically, the RACE method was carried out as follows. The 631R S6 primer (5'-AGCATCCTGTCTCCCTACTTCGTT-3', SEQ ID NO: 15) was prepared. PCR was performed using this primer and the Marathon-Ready™ cDNA derived from the human adult kidney (CLONTECH) as a template under the following
10 conditions: 94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 3 min;; and 72°C for 10 min, resulting in amplification of about 1.7 kbp cDNA fragment of the 3'-end. This fragment was subcloned into the pT7Blue-T vector by the TA cloning method to determine its
15 structure.

It became evident that human OCTN2 contains an open reading frame (ORF) encoding a protein consisting of 557 amino acid residues. Fig. 3 compares amino acid sequences of human OCTN1 and human OCTN2. Both showed overall amino
20 acid homology as high as about 76%. In addition, one consensus sequence (160 to 176) of sugar transporter was present in the amino acid sequence of human OCTN2 like human OCTN1. These facts indicated that human OCTN2 can be a novel transporter that is structurally related to human
25 OCTN1. Furthermore, a consensus sequence (218 to 225) of the ATP/GTP binding site was also present in the amino acid sequence of human OCTN2 like in human OCTN1.

Example 5 Northern analysis

30 Northern analysis was performed using about 900 bp human OCTN2 cDNA as a probe which was obtained by PCR with a set

of 631R S4 primer (5'-GTGCTGTTGGGCTCCTTCATTTCA-3', SEQ ID NO: 13) and 631R A1 primer (5'-AGCTGCATGAAGAGAAGGACACTG-3', SEQ ID NO: 14) in the same manner as for human OCTN1. The results are shown in Fig. 4. Although the expression pattern of human OCTN2 partly overlapped with that of human OCTN1, human OCTN2 differs from human OCTN1 in that the former was very intensely expressed in the kidney among fetal tissues, while the latter was strongly expressed also in cancer cell strains such as K-562, HeLa S3, SW480, etc. as well as the kidney, indicating that OCTN1 and OCTN2 may be involved in transport of substances such as carcinostatics in these cancer cells.

Example 6 Forced expression of human OCTN1 in human fetal kidney cells (HEK293) and its activity determination

Phage DNAs were extracted from positive phage clones obtained by screening the clones by the plaque hybridization method using the QIAGEN Lambda Kit (QIAGEN). After the DNA insert was subcloned into the pUC18 vector, cDNA containing the entire ORF which was cleaved out with SmaI and EcoRI was integrated between the EcoRI site and the blunted HindIII site of an expression vector for mammalian cells, pCDNA3 (Invitrogen), to obtain an expression plasmid DNA, pCDNA3/OCTN1. Plasmid DNA was prepared by alkaline-SDS method using the QIAGEN PLASMID MAXI Kit (QIAGEN).

The human fetal kidney-derived cell strain, HEK 293 cells were transfected with the plasmid pCDNA3/OCTN1 and pCDNA3 vector containing no insert as a control by the calcium phosphate method. First, the plasmid DNA (10 µg), a

Hepes buffer solution (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM Dextrose, and 21 mM Hepes pH 7.1) (1 ml), and 2 M CaCl₂ (62.5 µl) were combined and allowed to stand at room temperature for 30 min or more to form calcium phosphate coprecipitates. After cells were plated on 10-cm plates at 1.5 x 10⁶ cells per plate and cultured for 24 h, the calcium phosphate coprecipitates were added thereto, and the cells were further cultured for 24 h. Then, plates were washed with phosphate buffered saline (PBS), and the cells were further cultured for 24 h after the addition of fresh culture medium.

Transport experiment was performed using cells transfected with the plasmid DNA or untreated cells according to the following procedures. Cells were detached from plates using a rubber policeman, suspended in a transport buffer (containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM (+)-glucose, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 25 mM Hepes pH 7.4), and pre-incubated for 20 min. An appropriate amount of each labeled substrate ([¹⁴C]TEA (tetraethylammonium) (NEN), [³H]carnitine (L-carnitine hydrochloride) (Amersham), [³H]PCG (benzylpenicillin) (Amersham), [³H]quinidine (ARC), or [³H]pyrilamine (mepyramine) (Amersham)) was then added to the cell suspension, and the resulting mixture was incubated at 37°C for a predetermined period of time. Incubated cells were overlaid on a silicon layer prepared by layering a mixture of silicon oil and liquid paraffin (specific gravity = 1.022) on a 3 M KCl layer, and separated by centrifugation. Radioactivity of cells was measured to determine the into-the-cell transport activity. In this case, 1 x 10⁶ cells were used as one point of cells. HEK 293 cells were

cultured in Dulbecco's MEM containing 10% fetal calf serum (FCS) in an atmosphere of 5% carbon dioxide at 37°C.

First, the transporter capacity was measured in the cells transfected with pcDNA3/OCTN1 and untreated cells using TEA as a substrate (Fig. 5). A reaction time-dependent TEA uptake into the human OCTN1-transfected cells was clearly observed. This uptake was not observed in untreated cells. Next, effects of the addition of unlabeled TEA on the labeled substrate uptake in this system (cold inhibition) was examined (Fig. 6). A decrease in the apparent uptake of the labeled substrate was clearly seen depending on the concentration of cold TEA added. In this experiment, almost no uptake of the substrate into cells was observed in cells transfected with the pcDNA3 vector containing no insert (Mock) used as a control like in untreated cells used, clearly indicating that this uptake phenomenon is due to the transfection of the cells with human OCTN1. Next, to obtain the K_m (Michaelis constant) value of human OCTN1 to TEA, the uptake of ^{14}C -TEA with various concentrations was measured (Fig. 7). From Lineweaver-Burk reciprocal plot of the net uptake obtained by subtracting the amount of the uptake in Mock cells from that in the human OCTN1-transfected cells, the K_m value of 0.44 ± 0.04 mM was obtained with the maximal velocity, V_{max} of 6.68 ± 0.34 (nmol/3 min/mg). Next, the transport capacity of human OCTN1 for other substrate than TEA was examined (Fig. 8). When the transport capacity was measured using labeled organic cations such as labeled carnitine, quinidine, and pyrilamine, a significant increase in the uptake of these compounds was clearly observed in human OCTN1-transfected cells as compared with Mock cells,

clearly indicating that these organic cations can serve as substrates for human OCTN1. However, no significant increase in the uptake of an organic anion, PCG (benzylpenicillin), was observed.

5

Example 7 Activity measurement of human OCTN1 using
Xenopus oocytes

crRNA was synthesized *in vitro* using T7 RNA polymerase with pcDNA3/OCTN1 as a template. This crRNA was diluted to
10 the concentration of 0.3 ng/nl, and its 50-nl (15 ng) aliquot was injected into a single oocyte. As a control, 50 nl of distilled water was injected. These oocytes were cultured for 3 days, and then used for the transport experiment. After being preincubated in an uptake buffer
15 (0.05% Tween 80, 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM Hepes pH 7.4) at 25°C for 20 min, the oocytes were transferred to the uptake buffer containing 0.5 ml of labeled substrate to initiate the uptake. After the incubation at 25°C for 1 h, the oocytes were washed in
20 the ice-cold uptake buffer three times to terminate the reaction. The oocytes were solubilized in 5% SDS and mixed with Cleasol I (a cocktail for liquid scintillation counter) (3 ml) to determine the radioactivity. The radioactivity of the uptake buffer which contained the
25 labeled compound at the time of incubation (external solution) (10 µl) was also similarly measured. The ratio of the radioactivity (dpm value) in the oocytes to that (dpm value) in the external solution was used as the uptake activity.

Human OCTN1 also expresses the transport capacity for organic cations such as quinidine, mepyramine and carnitine, as well as TEA in this transport experiment system using *Xenopus* oocytes (Fig. 9).

5 Next, the transport capacity of human OCTN1 for carcinostatics, etc. was examined. The results revealed that human OCTN1 has the activity to transport actinomycin D, etoposide, vinblastine, and daunomycin (Fig. 10). These results strongly indicate that OCTN1 would be involved in
10 the into-the-cell translocation mechanism (mechanism for absorption by cells) for these drugs, which have been clinically used as carcinostatics. By designing and screening drugs utilizing the substrate specificity of OCTN1 so as to be readily recognized by this transporter,
15 it would be possible to efficiently develop useful drugs that can be readily absorbed by the cells.

Example 8 Forced expression of human OCTN2 in HEK cells and its activity measurement

20 The expression plasmid DNA for human OCTN2 in mammalian cells was prepared as follows.

A single-stranded cDNA was synthesized from poly(A)⁺ RNA derived from the human fetal kidney (CLONTECH) using the SuperScript™ II reverse transcriptase (GIBCO BRL). PCR was
25 performed using the thus-obtained cDNA as a template under the following conditions to amplify 5'- and 3'-end fragments of human OCTN2.

For the amplification of 5'-end fragment (about 800 bp) of human OCTN2, OCTN2 3 primer (5'-

30 GATGGATCCCGGACGGTCTTGGGTCGCCTGCTG-3', SEQ ID NO: 16) and

OCN2 4 primer (5'-GATGGATCCAAATGCTGCCACATAGTTGGAGAT-3',
SEQ ID NO: 17) were used. PCR was carried out using DNA
polymerase ExTaq (TaKaRa) and dNTPs (150 μ M 7-deaza dGTP,
50 μ M dGTP, 200 μ M dATP, 200 μ M dTTP, and 200 μ M dCTP)
5 according to the following conditions: 94°C for 2 min;
35 cycles of 94°C for 30 s, 63°C for 1 min, and 72°C for
2 min,; and 72°C for 10 min. For the amplification of 3'-
end fragment (about 1.2 kbp) of human OCTN2, OCTN2 7 primer
(5'-GATGGATCCATGGGCATGCAGACAGGCTTCAGC-3', SEQ ID NO: 18)
10 and OCTN2 8 primer (5'-GATGGATCCTTCCTCTTCAGTTTCTCCCTTACT-
3', SEQ ID NO: 19) were used. PCR was carried out using
DNA polymerase ExTaq (TaKaRa) and dNTPs (200 μ M dGTP, 200 μ M
dATP, 200 μ M dTTP, and 200 μ M dCTP) according to the
following conditions: 94°C for 2 min; 35 cycles of 94°C for
15 30 s, 63°C for 30 s, and 72°C for 2 min,; and 72°C for
10 min.

These fragments were respectively electrophoresed on
agarose gel, excised from the gel, purified, and subcloned
into the pT7Blue-T vector. Clones having no PCR error were
20 selected by sequencing, and clones from both fragments were
ligated at the PstI site in the overlapping region. Each
ligated fragment was eventually incorporated into the BamHI
site of the pcDNA3 vector, and used as the expression
plasmid DNA pcDNA3/OCTN2.

25 HEK cells were transfected with pcDNA3/OCTN2, the pcDNA3
vector containing no insert (Mock), or pcDNA3/OCTN1 by the
method described in Example 6 to perform transport
experiments. It was proved that human OCTN2 has a high
capacity to efficiently transport carnitine (Fig. 11). On
30 the other hand, human OCTN2 hardly transported TEA, which

were efficiently transported by human OCTN1, revealing that they clearly differ in their substrate specificities.

Next, Na⁺ dependence of human OCTN2-mediated carnitine transport was examined using a transport buffer in which Na⁺ was replaced with K⁺ (Fig. 12). The result showed that carnitine transport mediated by human OCTN2 completely depended on the presence of Na⁺, indicating that OCTN2 is a symport type transporter that transports substrates and Na⁺ in the same direction.

Example 9 Cloning of mouse OCTN1

Data base search using human OCTN1 cDNA sequence detected several Expressed Sequence Tags (ESTs) derived from mouse, which had very high homology to the human OCTN1 cDNA sequence. Based on these EST sequences, MONL 1 primer (5'-CGCGCCGAATCGCTGAATCCTTTC-3', SEQ ID NO: 20) and MONA 4 primer (5'-AGGCTTTTGATTTGTTCTGTTGAG-3', SEQ ID NO: 21) were prepared. PCR was performed using a set of these primers and cDNA prepared from poly(A)⁺ RNA derived from the mouse kidney as a template. As a result, fragments of about 2 kbp were amplified. These fragments were electrophoresed on agarose gels, excised from the gels, purified, and subcloned into the pT7Blue T vector (Novagen) by the TA cloning method. The sequence of mouse OCTN1 was determined by sequencing plural clones. The nucleotide sequence of cDNA thus determined is shown in SEQ ID NO: 23, and amino acid sequence of the protein encoded by the cDNA in SEQ ID NO: 22.

Example 10 Cloning of mouse OCTN2

First, MONB 20 primer (5'-CCCATGCCAACAAGGACAAAAAGC-3',
SEQ ID NO: 24) was prepared from the sequence of human
OCTN2 cDNA. The Marathon-Ready™ cDNA derived from the
5 mouse kidney (CLONTECH) was used as a template for the 5'-
Rapid Amplification of cDNA ends (RACE) to clone the 5'-end
sequence upstream of the primer. Next, data base search
was performed using human OCTN2 nucleotide sequence to
detect several ESTs derived from mouse, which had a very
10 high homology with human OCTN2. MONB 26 primer
(5'-ACAGAACAGAAAAGCCCTCAGTCA-3', SEQ ID NO: 25) was
prepared from these EST sequences. MONB 6 primer
(5'-TGTTTTTCGTGGGTGTGCTGATGG-3', SEQ ID NO: 26) was
prepared from the sequence obtained by the 5'-RACE. PCR
15 was performed using this primer and MONB 26 primer and cDNA
prepared from poly(A)⁺ RNA derived from the mouse kidney as
a template to amplify the 3'-end fragments. The sequence
of mouse OCTN2 was determined by sequencing directly of
after subcloning respective fragments. The nucleotide
20 sequence of the cDNA thus determined is shown in SEQ ID
NO: 28, and amino acid sequence of the protein encoded by
the cDNA in SEQ ID NO: 27.

Example 11 Tissue expression analysis of mouse OCTN1 and
25 mouse OCTN2

The expression amount of mouse OCTN1 and mouse OCTN2
genes in various tissues was examined by RT-PCR using a
mouse Multiple Tissue cDNA (MTC) panel (CLONTECH)
(Fig. 13). Primers used are MONL 1 and MONA 4 for mouse
30 OCTN1, and MONB 6 and MONB 26 for mouse OCTN2. As a

result, the high level expression of mouse OCTN1 was detected in the kidney and liver, while that of mouse OCTN2 in the kidney, liver, and 7-days old embryo.

5

Industrial Applicability

This invention provides a family of novel organic cation transporter genes and proteins encoded by these genes. Transporter proteins of this invention are useful for developing newly designed drugs that can be transported
10 mediated by these proteins, and pharmaceuticals for disorders caused by functional abnormalities of the proteins.

What is claimed is:

1 1. A substantially pure polypeptide comprising an amino
2 acid sequence at least 70% identical to any one of SEQ ID
3 NOs:1, 3, 22, or 27, wherein the polypeptide is a
4 transporter of an organic cation.

1 2. The polypeptide of claim 1, wherein the amino acid
2 sequence is at least 80% identical to any one of SEQ ID
3 NOs:1, 3, 22, or 27.

1 3. The polypeptide of claim 1, wherein the amino acid
2 sequence is at least 90% identical to any one of SEQ ID
3 NOs:1, 3, 22, or 27.

1 4. The polypeptide of claim 1, wherein the amino acid
2 sequence is at least 95% identical to any one of SEQ ID
3 NOs:1, 3, 22, or 27.

1 5. A substantially pure polypeptide comprising the
2 sequence of any one of SEQ ID NOs:1, 3, 22, or 27.

1 6. A substantially pure polypeptide comprising the
2 amino acid sequence of any one of SEQ ID NOs:1, 3, 22, or
3 27, with up to 30 conservative amino acid substitutions,
4 wherein the polypeptide is a transporter of an organic
5 cation.

1 7. A substantially pure polypeptide encoded by a
2 nucleic acid that hybridizes under stringent conditions to

3 a probe the sequence of which consists of any one of SEQ ID
4 NOs:2, 4, 23, or 28, wherein the polypeptide is a
5 transporter of an organic cation.

1 8. An isolated nucleic acid encoding the polypeptide of
2 claim 1.

1 9. An isolated nucleic acid encoding the polypeptide of
2 claim 5.

1 10. An isolated nucleic acid encoding the polypeptide
2 of claim 6.

1 11. An isolated nucleic acid comprising a strand that
2 hybridizes under stringent conditions to a single stranded
3 probe, the sequence of which consists of any one of SEQ ID
4 NOs:2, 4, 23, or 28, or the complement of any one of SEQ ID
5 NOs:2, 4, 23, or 28.

1 12. The isolated nucleic acid of claim 11, wherein the
2 nucleic acid encodes a polypeptide that is a transporter of
3 an organic cation.

1 13. The nucleic acid of claim 12, wherein the amino
2 acid sequence of the polypeptide comprises any one of SEQ
3 ID NOs: 1, 3, 22, or 27.

1 14. The nucleic acid of claim 11, wherein the strand is
2 at least 15 nucleotides in length.

1 15. The nucleic acid of claim 14, wherein the nucleic
2 acid is an antisense nucleic acid that inhibits expression
3 of a polypeptide comprising any one of SEQ ID NOs: 1, 3,
4 22, or 27.

1 16. A vector comprising the nucleic acid of claim 8.

1 17. A vector comprising the nucleic acid of claim 9.

1 18. A vector comprising the nucleic acid of claim 10.

1 19. A vector comprising the nucleic acid of claim 11.

1 20. A vector comprising the nucleic acid of claim 12.

1 21. A cultured host cell comprising the nucleic acid of
2 claim 8.

1 22. A cultured host cell comprising the nucleic acid of
2 claim 9.

1 23. A cultured host cell comprising the nucleic acid of
2 claim 10.

1 24. A cultured host cell comprising the nucleic acid of
2 claim 11.

1 25. A cultured host cell comprising the nucleic acid of
2 claim 12.

1 26. An antibody that specifically binds to the
2 polypeptide of claim 1.

1 27. A method of producing a polypeptide, the method
2 comprising isolating the polypeptide from the cultured host
3 cell of claim 21.

1 28. The polypeptide of claim 1, wherein the polypeptide
2 comprises the sequence Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa6- Xaa7-
3 Gly-Arg-Xaa8-Xaa9-Xaa10-Xaa11-Xaa12, wherein

4 Xaa1 is Leu, Ile, Val, Met, Ser, Thr, Ala, or Gly;

5 Xaa2 is Leu, Ile, Val, Met, Phe, Ser, Ala, or Gly;

6 Xaa3 is any amino acid;

7 Xaa4 is Leu, Ile, Val, Met, Ser, Ala Xaa5 is Asp or Glu;

8 Xaa6 is any amino acid;

9 Xaa7 is Leu, Ile, Val, Met, Phe, Tyr, Trp, or Ala;

10 Xaa8 is Arg or Lys;

11 Xaa9 is any amino acid;

12 Xaa10 is any amino acid;

- 13 Xaa11 is any amino acid; and
- 14 Xaa12 is Gly, Ser, Thr, or Ala.

TRANSPORTER GENES

Abstract of the Disclosure

Novel genes significantly homologous to organic cation
transporters OCT1 and OCT2 have been successfully isolated
5 by screening a fetal gene library by random sequencing.
Proteins encoded by these genes function as transporters of
various organic cations.

10

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Figure 1

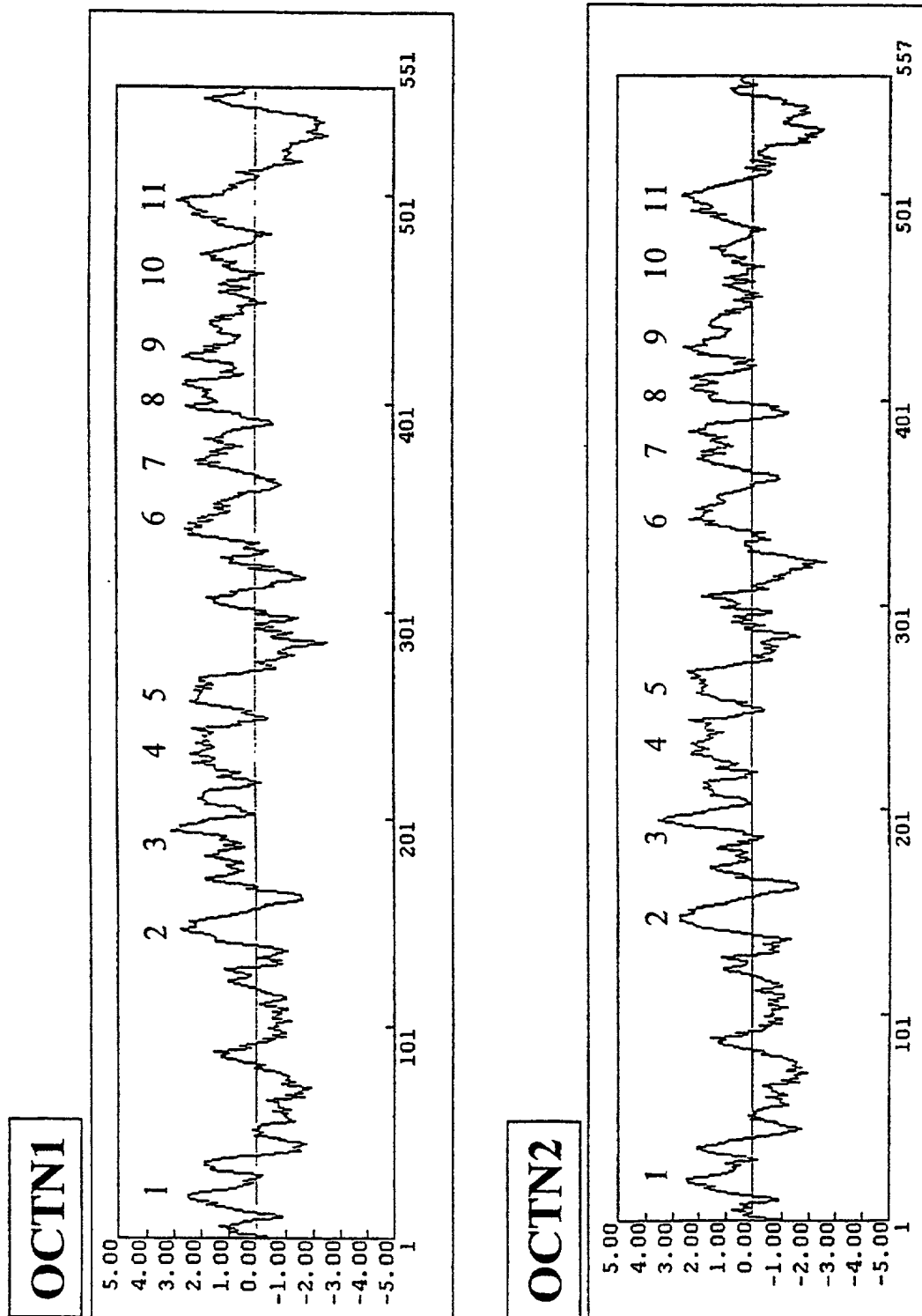


Figure 2

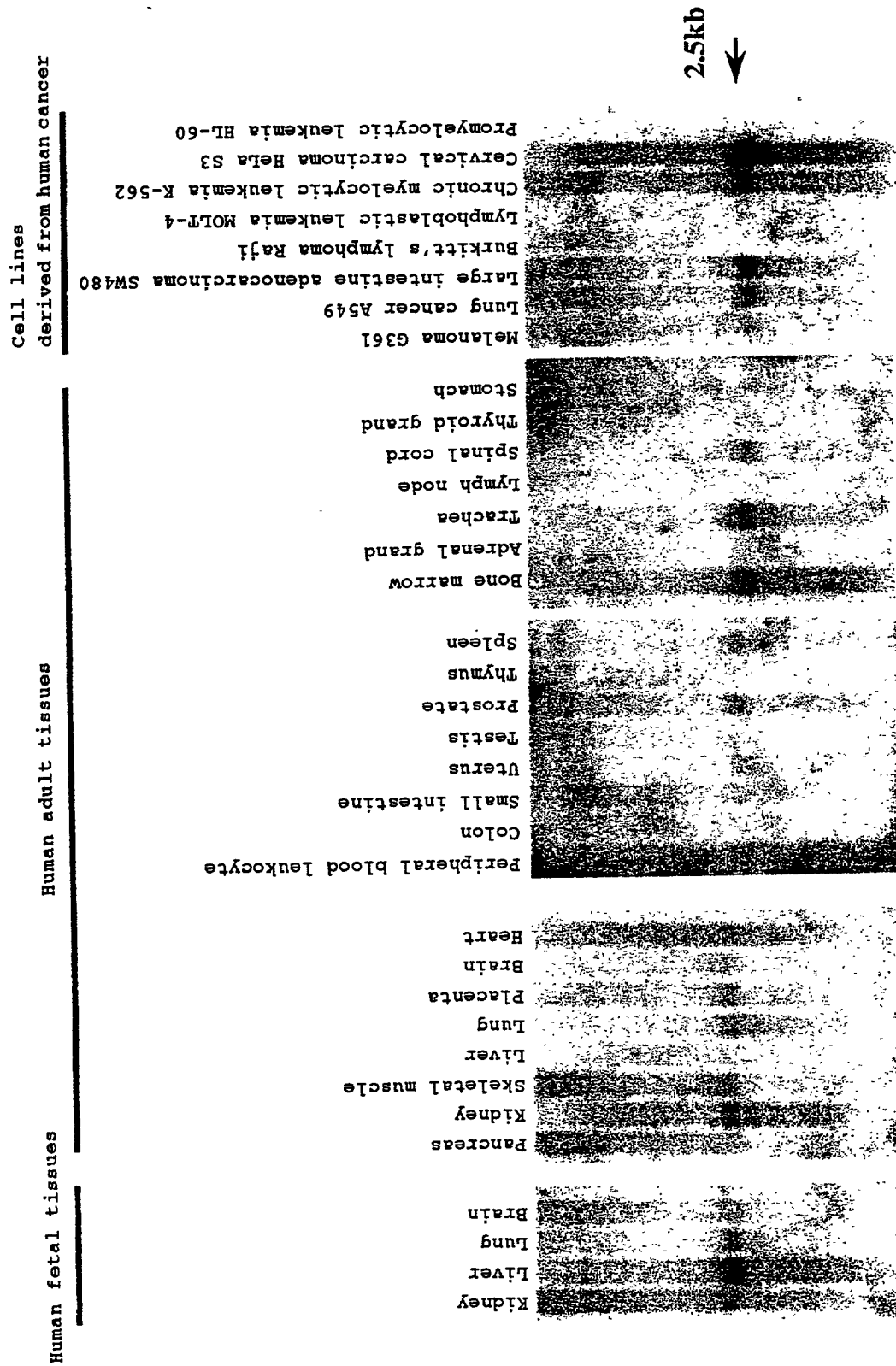


Figure 3

| | | | | | | | | | | | |
|-------|-----|------------|-------------|------------|------------|------------|------------|------------|-------------|-------------|------------|
| OCTN1 | 1 | MRDYDAIAT | UCBQGRFORI | TEFFISASTI | PACHNOMEVV | ELAGIRIRRG | RVEDANISS | AVRNNSMEIR | IRKGRBYPHS | ISQVRJATIA | NFSALGLEPG |
| OCTN2 | 1 | MRDYDEVAT | ECBQGRFORI | TEFFISASTI | PNGTQLSSY | ELIATIRIRG | RVEDANISS | AVRNHTIIR | IRDCGRBYPHS | GRARATATIA | NFSALGLEPG |
| OCTN1 | 101 | RDVDEGQLEQ | ESQDQTEES | QDVYESTIVT | ENLVGQRONY | KVPLTNSIRP | GVYETGSRVS | GOESDRGRK | NVLEATIAVQ | IGESRIGQIFS | ISWEMTVLFR |
| OCTN2 | 101 | RDVDEGQLEQ | ESQDQTEES | QDVYESTIVT | ENLVGQEDY | KAPETLSIRP | GVYETGSHIS | GOESDRGRK | NVLEVTGCMQ | IGESRIGQIFS | KNFEMFYVLR |
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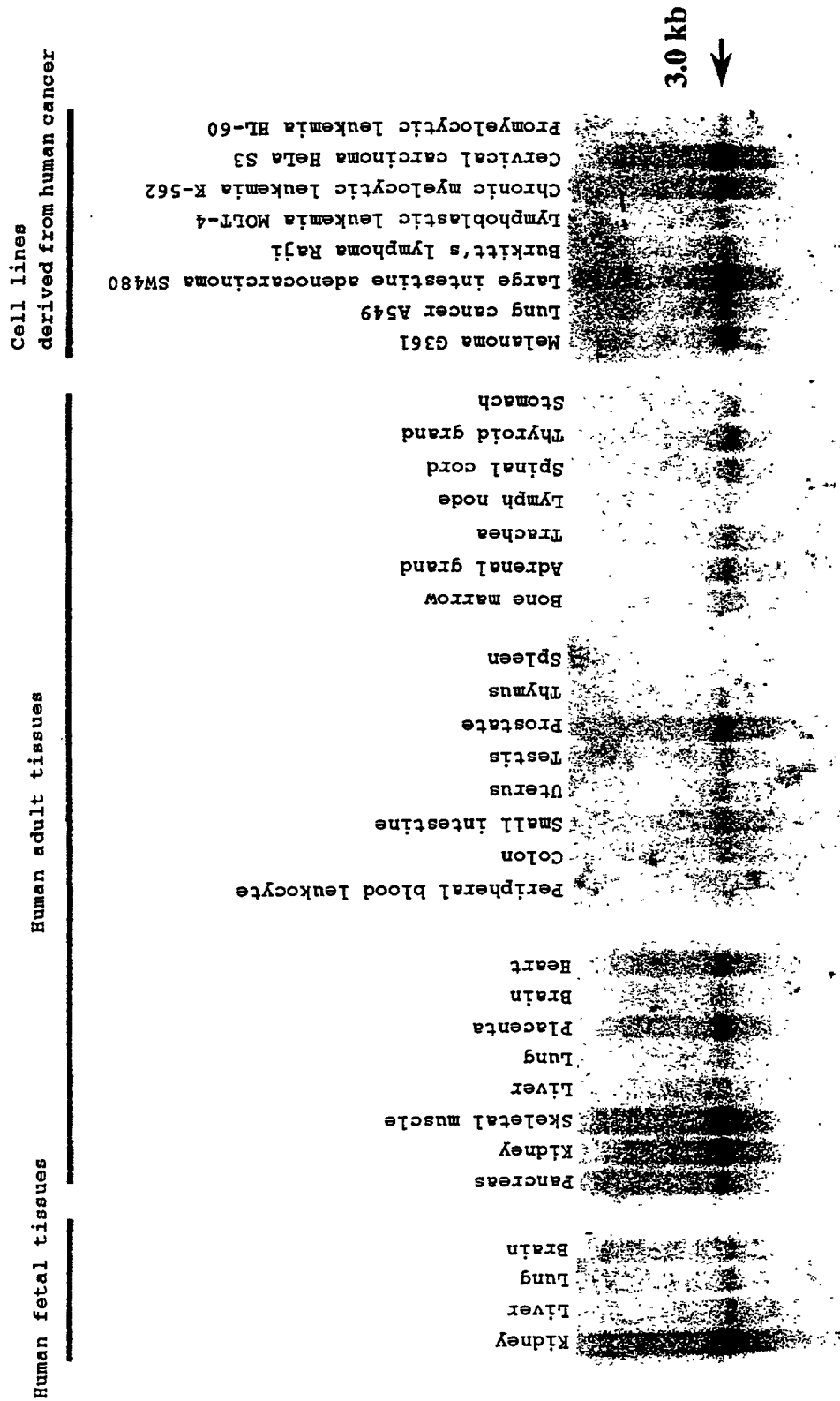


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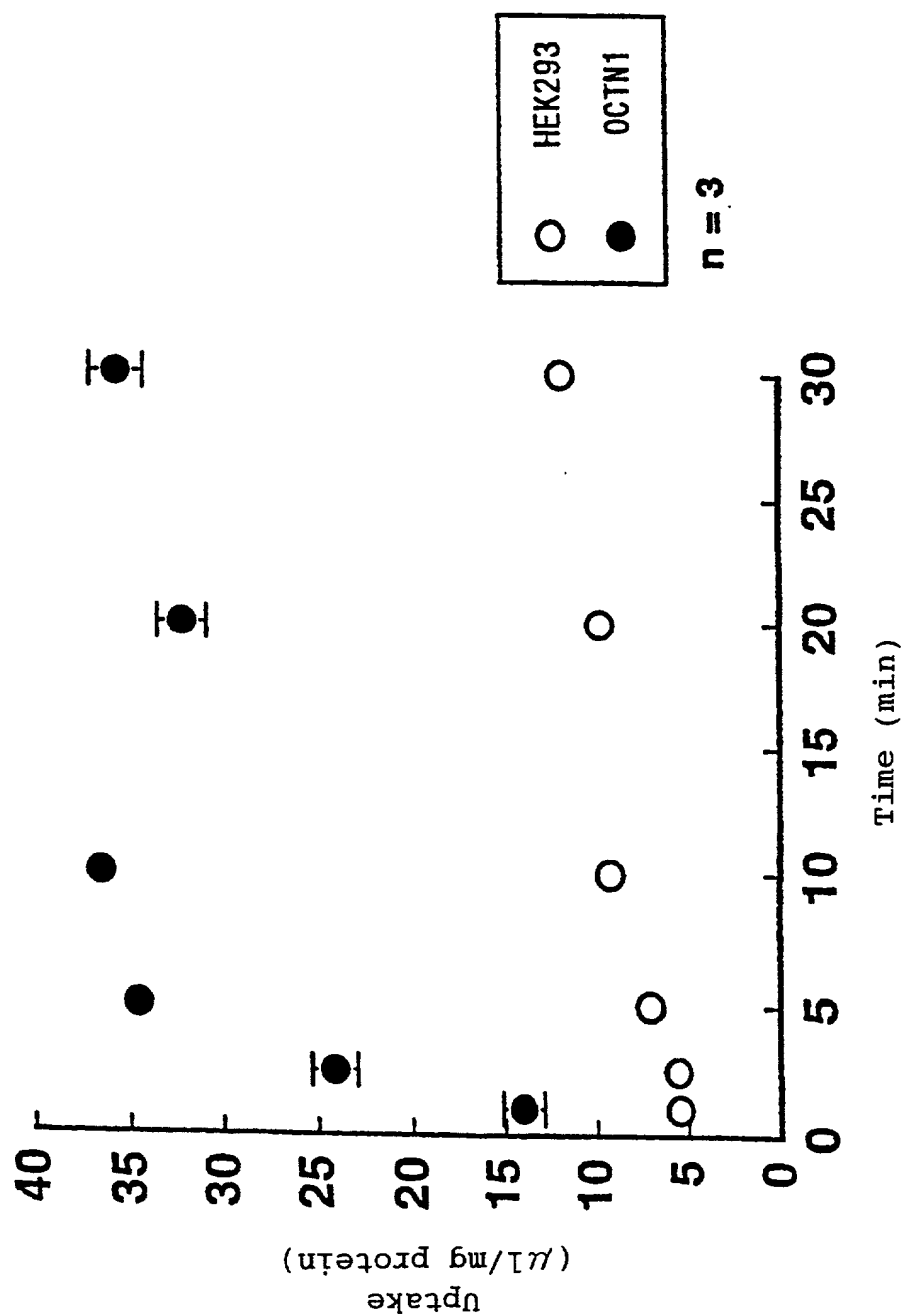


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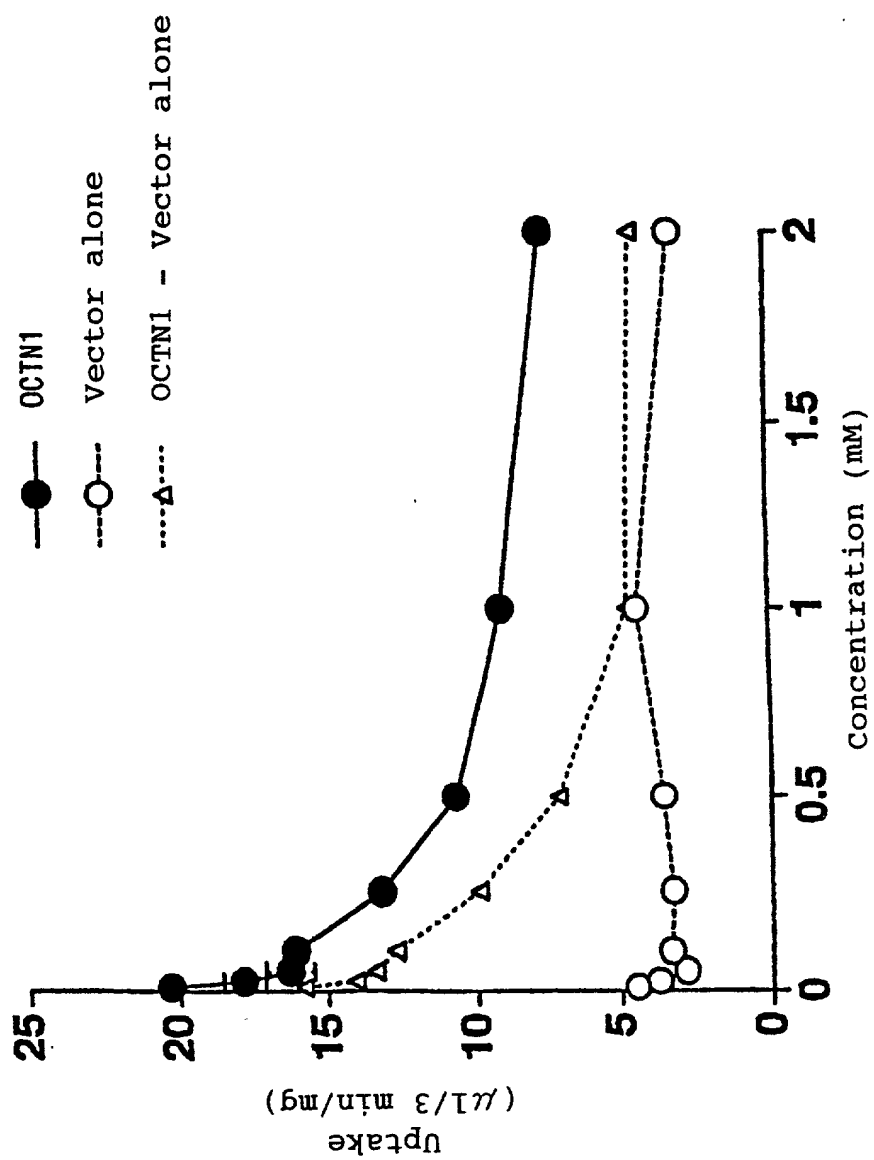


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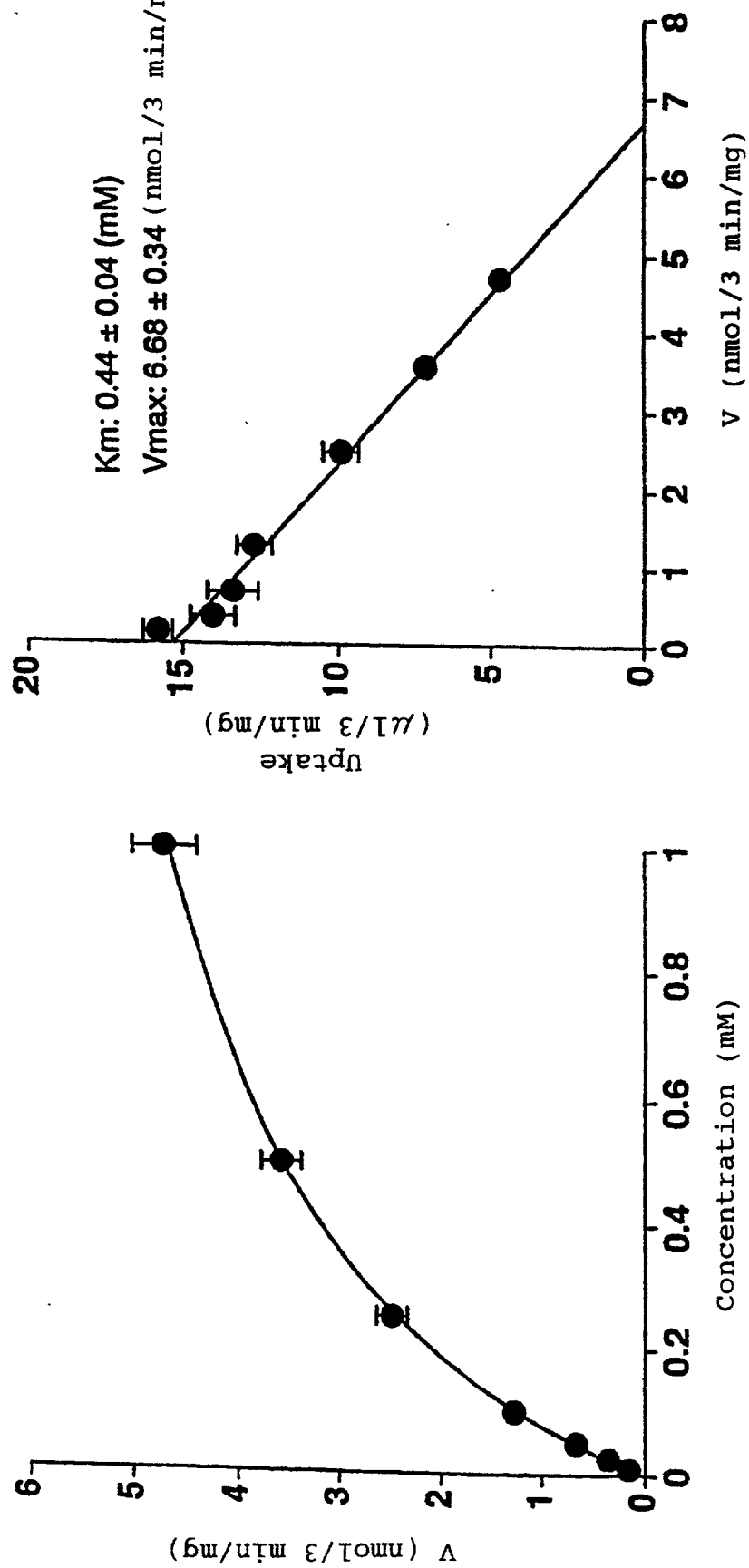


Figure 8



Figure 9

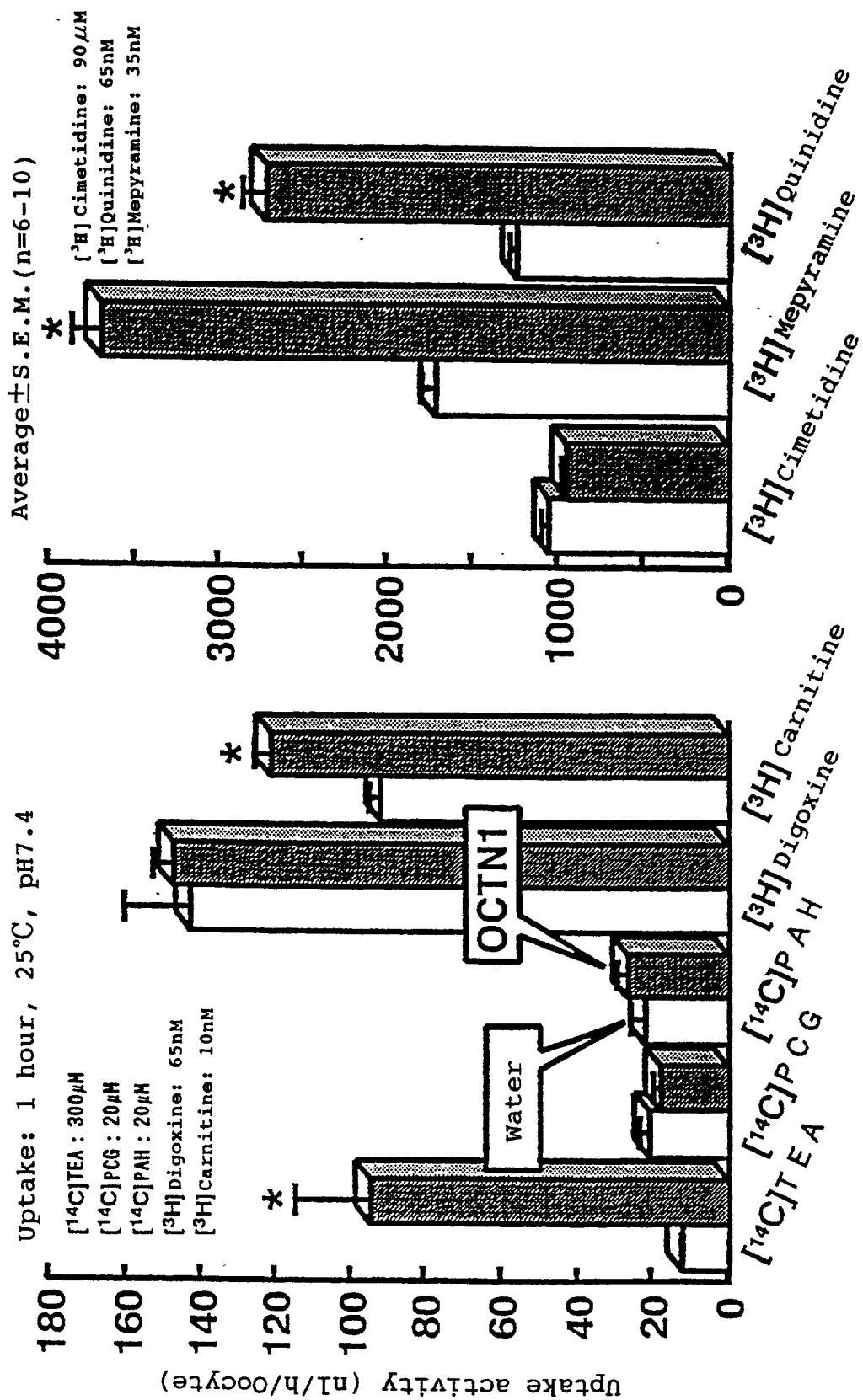


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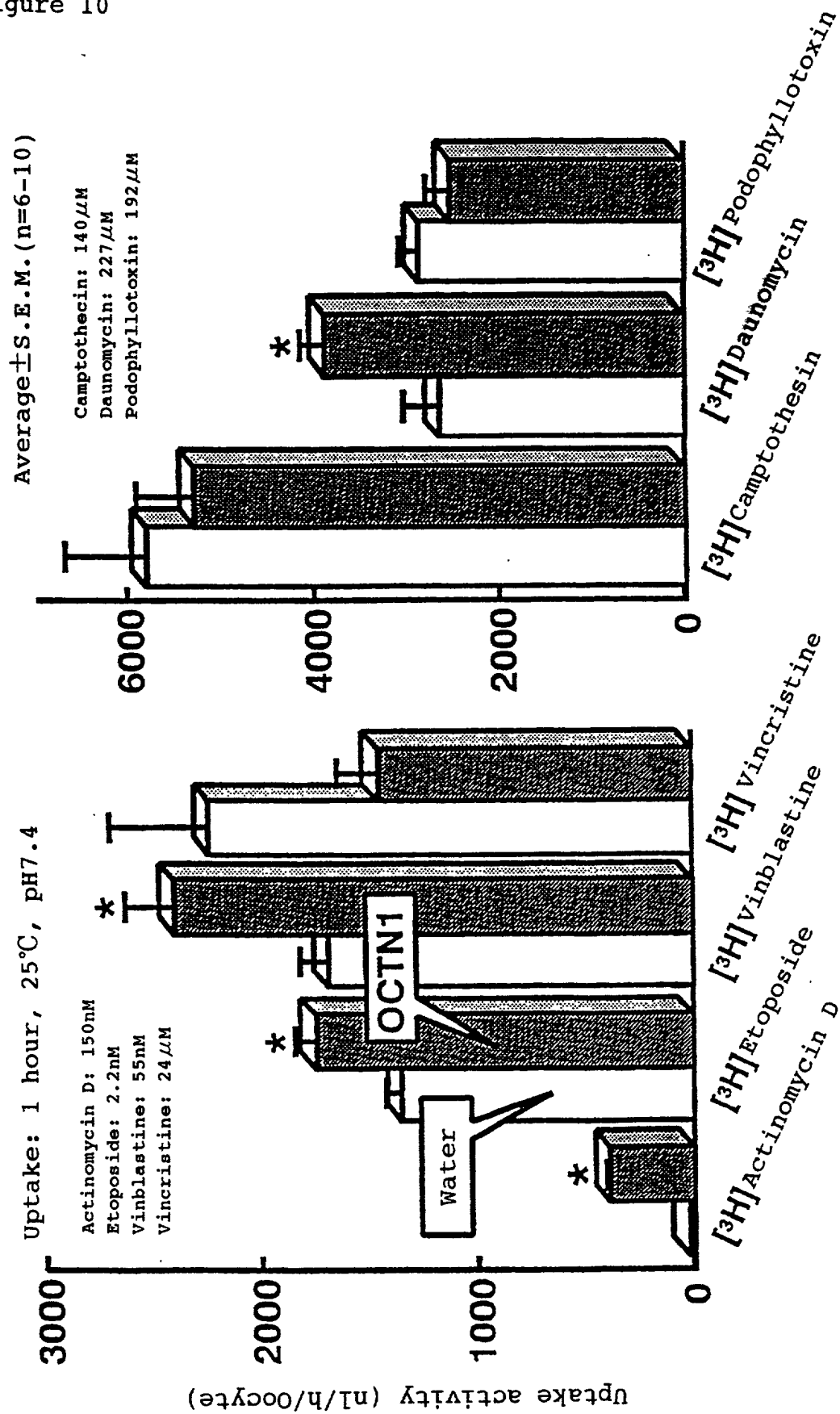
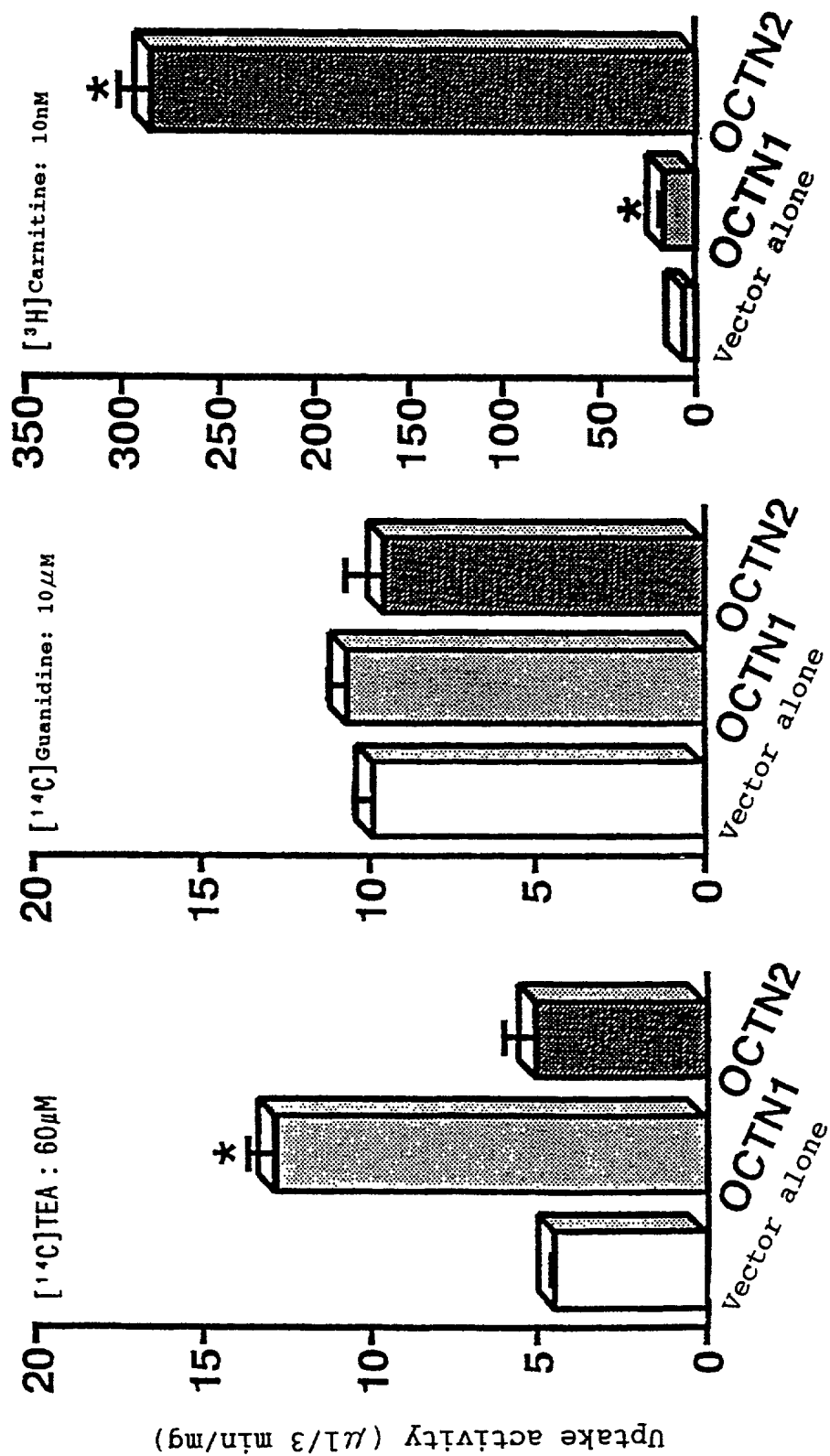


Figure 11



Uptake: 3 min, 37°C, pH7.4

Average ± S.E.M. (n=3)

Figure 12

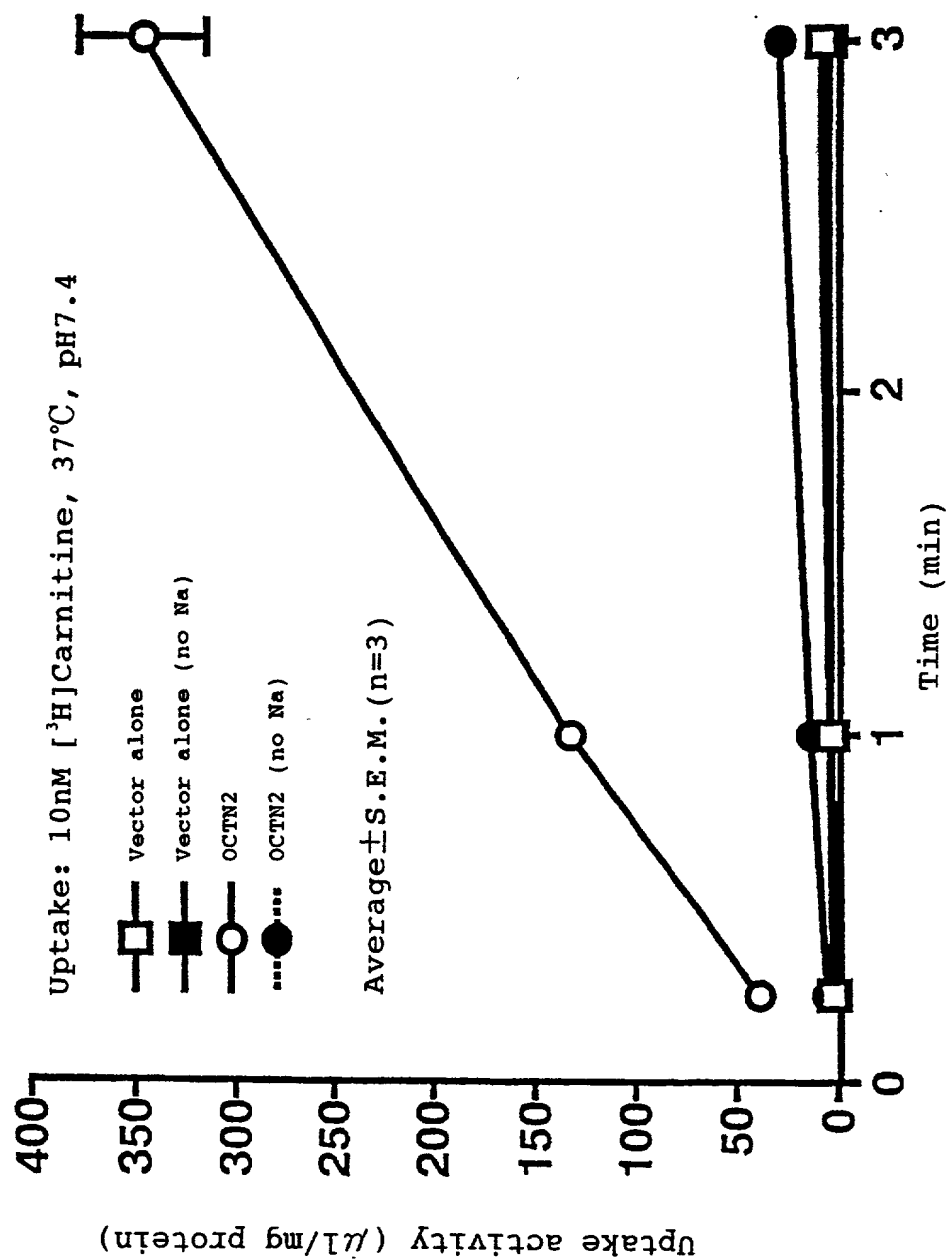


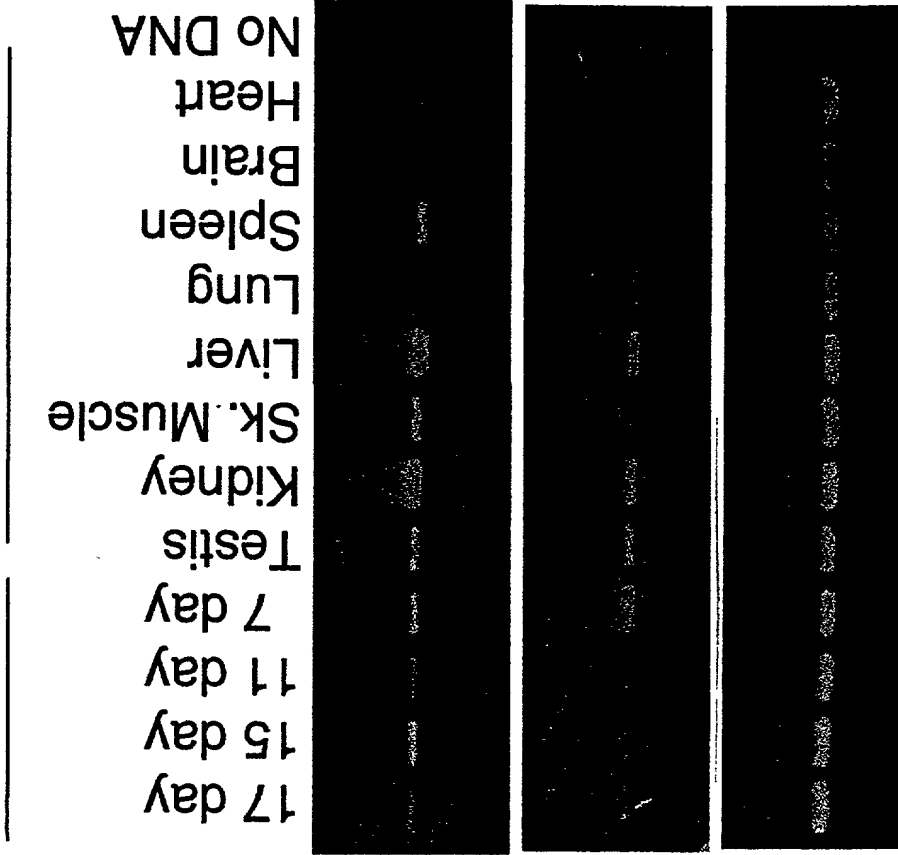
Figure 13

mouse OCTN 1

mouse OCTN 2

G3PDH

Embryo Adult Tissues



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled TRANSPORTER GENES, the specification of which:

- ☒ is attached hereto.
☐ was filed on _ as Application Serial No. _ and was amended on _____.
☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

| U.S. Serial No. | Filing Date | Status |
|-----------------|-------------------|---------|
| PCT/JP98/04009 | September 7, 1998 | Pending |

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

| Country | Application No. | Filing Date | Priority Claimed |
|---------|-----------------|-------------------|---|
| Japan | 9/260972 | September 8, 1997 | <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No |
| Japan | 10/156660 | May 20, 1998 | <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No |

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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|----------------------------------|------------------------------------|
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| John T. Li, Reg. 44,210 | J. Peter Fasse, Reg. 32,983 |
| John W. Freeman, Reg. 29,066 | Timothy A. French, Reg. 30,175 |
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Combined Declaration and Power of Attorney

Page 2 of 2 Pages

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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SEQUENCE LISTING

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 Gly Val Ser Ser Thr Ala Ser Arg Leu Gly Ser Ile Leu Ser Pro Tyr
 465 470 475

ttc gtt tac ctt ggt gcc tac gac cgc ttc ctg ccc tac att ctc atg 1608
 Phe Val Tyr Leu Gly Ala Tyr Asp Arg Phe Leu Pro Tyr Ile Leu Met
 480 485 490 495

gga agt ctg acc atc ctg aca gcc atc ctc acc ttg ttt ctc cca gag 1656
 Gly Ser Leu Thr Ile Leu Thr Ala Ile Leu Thr Leu Phe Leu Pro Glu
 500 505 510

agc ttc ggt acc cca ctc cca gac acc att gac cag atg cta aga gtc 1704
 Ser Phe Gly Thr Pro Leu Pro Asp Thr Ile Asp Gln Met Leu Arg Val
 515 520 525

aaa gga atg aaa cac aga aaa act cca agt cac aca agg atg tta aaa 1752
 Lys Gly Met Lys His Arg Lys Thr Pro Ser His Thr Arg Met Leu Lys
 530 535 540

gat ggt caa gaa agg ccc aca atc ctt aaa agc aca gcc ttc 1794
 Asp Gly Gln Glu Arg Pro Thr Ile Leu Lys Ser Thr Ala Phe
 545 550 555

taacatcgct tccagtaagg gagaaactga agaggaa

1831

<210> 5

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

<400> 5

ctaatacgac tcactatagg gc

22

<210> 6

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

<400> 6

tgtagcgtga agacgacaga a

21

<210> 7

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

<400> 7

tcgagcggcc gcccgggcag gt

22

<210> 8

<211> 22

<212> DNA

<213> Artificial Sequence

CCDC1036.1

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

agggcggtggt gcggagggcg gt 22

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

cttttgagca agttcagcct 20

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

agaggtggct tatgagtatt tctt 24

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

ccaggggtttt cccagtcacg ac 22

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

22

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

24

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

24

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

<400> 15

agcatcctgt ctccctactt cgtt 24

<210> 16

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

<400> 16

gatggatccc ggacggtctt gggtcgcctg ctg 33

<210> 17

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

<400> 17

gatggatcca aatgctgcca catagttgga gat 33

<210> 18

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

<400> 18

gatggatcca tgggcatgca gacaggcttc agc 33

<210> 19

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

gatggatcct tcctcttcag tttctccctt act 33

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

cgcgccgaat cgctgaatcc tttc 24

<213> Artificial Sequence

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

aggcttttga tttgttctgt tgag 24

<213> Mus musculus

Met Arg Asp Tyr Asp Glu Val Ile Ala Phe Leu Gly Glu Trp Gly Pro

| | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|--|
| 1 | | | | 5 | | | | | 10 | | | | | 15 | | | |
| Phe | Gln | Arg | Leu | Ile | Phe | Phe | Leu | Leu | Ser | Ala | Ser | Ile | Ile | Pro | Asn | | |
| | | | 20 | | | | | 25 | | | | | 30 | | | | |
| Gly | Phe | Asn | Gly | Met | Ser | Val | Val | Phe | Leu | Ala | Gly | Thr | Pro | Glu | His | | |
| | | 35 | | | | | 40 | | | | | 45 | | | | | |
| Arg | Cys | Leu | Val | Pro | Asp | Thr | Val | Asn | Leu | Ser | Ser | Ser | Trp | Arg | Asn | | |
| | 50 | | | | | 55 | | | | | 60 | | | | | | |
| His | Ser | Ile | Pro | Leu | Glu | Thr | Lys | Asp | Gly | Arg | Gln | Val | Pro | Gln | Ser | | |
| 65 | | | | | 70 | | | | | 75 | | | | | 80 | | |
| Cys | Arg | Arg | Tyr | Arg | Leu | Ala | Thr | Ile | Ala | Asn | Phe | Ser | Ala | Met | Gly | | |
| | | | | 85 | | | | | 90 | | | | | 95 | | | |
| Leu | Glu | Pro | Gly | Gln | Asp | Val | Asp | Leu | Glu | Gln | Leu | Glu | Gln | Glu | Ser | | |
| | | | 100 | | | | | 105 | | | | | 110 | | | | |
| Cys | Leu | Asp | Gly | Trp | Glu | Tyr | Asp | Lys | Asp | Ile | Phe | Leu | Ser | Thr | Ile | | |
| | | 115 | | | | | 120 | | | | | 125 | | | | | |
| Val | Thr | Glu | Trp | Asn | Leu | Val | Cys | Glu | Asp | Asp | Trp | Lys | Thr | Pro | Leu | | |
| | 130 | | | | | 135 | | | | | 140 | | | | | | |
| Thr | Thr | Ser | Leu | Phe | Phe | Val | Gly | Val | Leu | Cys | Gly | Ser | Phe | Val | Ser | | |
| 145 | | | | | 150 | | | | | 155 | | | | | 160 | | |
| Gly | Gln | Leu | Ser | Asp | Arg | Phe | Gly | Arg | Lys | Lys | Val | Leu | Phe | Ala | Thr | | |
| | | | | 165 | | | | | 170 | | | | | 175 | | | |
| Met | Ala | Val | Gln | Thr | Gly | Phe | Ser | Phe | Val | Gln | Ile | Phe | Ser | Thr | Asn | | |
| | | | 180 | | | | | 185 | | | | | 190 | | | | |
| Trp | Glu | Met | Phe | Thr | Val | Leu | Phe | Ala | Ile | Val | Gly | Met | Gly | Gln | Ile | | |
| | | 195 | | | | | 200 | | | | | 205 | | | | | |
| Ser | Asn | Tyr | Val | Val | Ala | Phe | Ile | Leu | Gly | Thr | Glu | Ile | Leu | Ser | Lys | | |
| | 210 | | | | | 215 | | | | | 220 | | | | | | |
| Ser | Val | Arg | Ile | Ile | Phe | Ser | Thr | Leu | Gly | Val | Cys | Thr | Phe | Phe | Ala | | |
| 225 | | | | | 230 | | | | | 235 | | | | | 240 | | |

| | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| Ile | Gly | Tyr | Met | Val | Leu | Pro | Leu | Phe | Ala | Tyr | Phe | Ile | Arg | Asp | Trp | |
| | | | 245 | | | | | | 250 | | | | | 255 | | |
| Arg | Met | Leu | Leu | Leu | Ala | Leu | Thr | Leu | Pro | Gly | Leu | Phe | Cys | Val | Pro | |
| | | | 260 | | | | | 265 | | | | | 270 | | | |
| Leu | Trp | Trp | Phe | Ile | Pro | Glu | Ser | Pro | Arg | Trp | Leu | Ile | Ser | Gln | Arg | |
| | | 275 | | | | | 280 | | | | | 285 | | | | |
| Arg | Phe | Ala | Glu | Ala | Glu | Gln | Ile | Ile | Gln | Lys | Ala | Ala | Lys | Met | Asn | |
| | 290 | | | | | 295 | | | | | 300 | | | | | |
| Ser | Ile | Val | Ala | Pro | Ala | Gly | Ile | Phe | Asp | Pro | Leu | Glu | Leu | Gln | Glu | |
| 305 | | | | | 310 | | | | | 315 | | | | | 320 | |
| Leu | Asn | Ser | Leu | Lys | Gln | Gln | Lys | Val | Ile | Ile | Leu | Asp | Leu | Phe | Arg | |
| | | | 325 | | | | | | 330 | | | | | 335 | | |
| Thr | Arg | Asn | Ile | Ala | Thr | Ile | Thr | Val | Met | Ala | Val | Met | Leu | Trp | Met | |
| | | 340 | | | | | | 345 | | | | | 350 | | | |
| Leu | Thr | Ser | Val | Gly | Tyr | Phe | Ala | Leu | Ser | Leu | Asn | Val | Pro | Asn | Leu | |
| | | 355 | | | | | 360 | | | | | 365 | | | | |
| His | Gly | Asp | Val | Tyr | Leu | Asn | Cys | Phe | Leu | Ser | Gly | Leu | Ile | Glu | Val | |
| | 370 | | | | | 375 | | | | | 380 | | | | | |
| Pro | Ala | Tyr | Phe | Thr | Ala | Trp | Leu | Leu | Leu | Arg | Thr | Leu | Pro | Arg | Arg | |
| 385 | | | | | 390 | | | | | 395 | | | | | 400 | |
| Tyr | Ile | Ile | Ala | Gly | Val | Leu | Phe | Trp | Gly | Gly | Gly | Val | Leu | Leu | Leu | |
| | | | 405 | | | | | | 410 | | | | | 415 | | |
| Ile | Gln | Val | Val | Pro | Glu | Asp | Tyr | Asn | Phe | Val | Ser | Ile | Gly | Leu | Val | |
| | | 420 | | | | | | 425 | | | | | 430 | | | |
| Met | Leu | Gly | Lys | Phe | Gly | Ile | Thr | Ser | Ala | Phe | Ser | Met | Leu | Tyr | Val | |
| | | 435 | | | | | 440 | | | | | 445 | | | | |
| Phe | Thr | Ala | Glu | Leu | Tyr | Pro | Thr | Leu | Val | Arg | Asn | Met | Ala | Val | Gly | |
| | 450 | | | | | 455 | | | | | 460 | | | | | |

Ile Thr Ser Met Ala Ser Arg Val Gly Ser Ile Ile Ala Pro Tyr Phe
465 470 475 480

Val Tyr Leu Gly Ala Tyr Asn Arg Leu Leu Pro Tyr Ile Leu Met Gly
485 490 495

Ser Leu Thr Val Leu Ile Gly Ile Ile Thr Leu Phe Phe Pro Glu Ser
500 505 510

Phe Gly Val Thr Leu Pro Glu Asn Leu Glu Gln Met Gln Lys Val Arg
515 520 525

Gly Phe Arg Cys Gly Lys Lys Ser Thr Val Ser Val Asp Arg Glu Glu
530 535 540

Ser Pro Lys Val Leu Ile Thr Ala Phe
545 550

<210> 23

<211> 2083

<212> DNA

<213> Mus musculus

<220>

<221> CDS

<222> (122)..(1780)

<400> 23

attcggcaca ggacggcgtg ttgacgagc cacctaggaa gatcccctca gcgcgccgaa 60

tcgctgaatc ctttctctcc acccacctcc ctcacgcaag ctgaggagga gaggtggaaa 120

c atg cgg gac tac gac gag gtg atc gcc ttc ctg ggc gag tgg ggg ccc 169

Met Arg Asp Tyr Asp Glu Val Ile Ala Phe Leu Gly Glu Trp Gly Pro

1

5

10

15

ttc cag cgc ctc atc ttc ttt ctg ctc agc gcc agc atc atc ccc aat 217

Phe Gln Arg Leu Ile Phe Phe Leu Leu Ser Ala Ser Ile Ile Pro Asn

20

25

30

ggc ttc aat ggt atg tca gtc gtg ttc ctg gcg ggg acc ccc gag cac 265

Gly Phe Asn Gly Met Ser Val Val Phe Leu Ala Gly Thr Pro Glu His

35

40

45

002000-000000

| | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| cgt | tgc | ctg | gtt | cct | gac | act | gtg | aac | ctg | agc | agc | tcc | tgg | cgc | aac | 313 |
| Arg | Cys | Leu | Val | Pro | Asp | Thr | Val | Asn | Leu | Ser | Ser | Ser | Trp | Arg | Asn | |
| | 50 | | | | | 55 | | | | | 60 | | | | | |
| cac | agc | atc | ccc | ttg | gag | acg | aag | gac | gga | cga | cag | gtg | cct | cag | agc | 361 |
| His | Ser | Ile | Pro | Leu | Glu | Thr | Lys | Asp | Gly | Arg | Gln | Val | Pro | Gln | Ser | |
| | 65 | | | | 70 | | | | 75 | | | | | | 80 | |
| tgc | cgc | cgc | tac | cga | ctg | gcc | acc | atc | gcc | aac | ttc | tct | gcg | atg | ggg | 409 |
| Cys | Arg | Arg | Tyr | Arg | Leu | Ala | Thr | Ile | Ala | Asn | Phe | Ser | Ala | Met | Gly | |
| | | | | 85 | | | | | 90 | | | | | 95 | | |
| ctg | gag | cca | gga | cag | gac | gtg | gat | ctg | gag | cag | ctg | gag | cag | gag | agc | 457 |
| Leu | Glu | Pro | Gly | Gln | Asp | Val | Asp | Leu | Glu | Gln | Leu | Glu | Gln | Glu | Ser | |
| | | | 100 | | | | | 105 | | | | | 110 | | | |
| tgc | ctg | gat | ggc | tgg | gag | tac | gac | aag | gac | atc | ttc | ctg | tcc | acc | atc | 505 |
| Cys | Leu | Asp | Gly | Trp | Glu | Tyr | Asp | Lys | Asp | Ile | Phe | Leu | Ser | Thr | Ile | |
| | | 115 | | | | | 120 | | | | | 125 | | | | |
| gtg | aca | gag | tgg | aat | ctg | gtg | tgt | gag | gat | gac | tgg | aag | aca | ccc | ctc | 553 |
| Val | Thr | Glu | Trp | Asn | Leu | Val | Cys | Glu | Asp | Asp | Trp | Lys | Thr | Pro | Leu | |
| | 130 | | | | | 135 | | | | | 140 | | | | | |
| acc | acc | tcc | ctg | ttc | ttc | gta | ggc | gtt | ctc | tgc | ggc | tcc | ttc | gtg | tct | 601 |
| Thr | Thr | Ser | Leu | Phe | Phe | Val | Gly | Val | Leu | Cys | Gly | Ser | Phe | Val | Ser | |
| | 145 | | | | 150 | | | | | 155 | | | | | 160 | |
| ggg | cag | ctg | tca | gac | agg | ttt | ggc | agg | aag | aaa | gtc | ctc | ttt | gca | acc | 649 |
| Gly | Gln | Leu | Ser | Asp | Arg | Phe | Gly | Arg | Lys | Lys | Val | Leu | Phe | Ala | Thr | |
| | | | | 165 | | | | | 170 | | | | | 175 | | |
| atg | gct | gtg | cag | act | gga | ttc | agc | ttc | gtg | cag | att | ttc | tca | acc | aac | 697 |
| Met | Ala | Val | Gln | Thr | Gly | Phe | Ser | Phe | Val | Gln | Ile | Phe | Ser | Thr | Asn | |
| | | | 180 | | | | | 185 | | | | | 190 | | | |
| tgg | gag | atg | ttc | act | gtg | ttg | ttt | gcc | att | gtg | ggc | atg | ggc | cag | atc | 745 |
| Trp | Glu | Met | Phe | Thr | Val | Leu | Phe | Ala | Ile | Val | Gly | Met | Gly | Gln | Ile | |
| | | 195 | | | | | 200 | | | | | 205 | | | | |
| tcc | aac | tac | gtg | gtg | gcc | ttc | ata | cta | gga | act | gaa | atc | ctg | agc | aag | 793 |
| Ser | Asn | Tyr | Val | Val | Ala | Phe | Ile | Leu | Gly | Thr | Glu | Ile | Leu | Ser | Lys | |
| | 210 | | | | | 215 | | | | | 220 | | | | | |

atc ggc tac atg gtc ctg ccg ctg ttt gca tac ttc atc aga gac tgg 889
Ile Gly Tyr Met Val Leu Pro Leu Phe Ala Tyr Phe Ile Arg Asp Trp
245 250 255

agg atg ctg ctg ctg gcc ctg aca ctg cct ggc ctg ttc tgt gtt ccc 937
Arg Met Leu Leu Leu Ala Leu Thr Leu Pro Gly Leu Phe Cys Val Pro
260 265 270

ctg tgg tgg ttt att cca gaa tct ccc cgg tgg ctg ata tcc cag agg 985
Leu Trp Trp Phe Ile Pro Glu Ser Pro Arg Trp Leu Ile Ser Gln Arg
275 280 285

aga ttt gca gag gcc gaa cag atc atc cag aaa gcc gca aag atg aac 1033
Arg Phe Ala Glu Ala Glu Gln Ile Ile Gln Lys Ala Ala Lys Met Asn
290 295 300

agc atc gtg gcg cca gca ggg ata ttc gat cct cta gag cta cag gag 1081
Ser Ile Val Ala Pro Ala Gly Ile Phe Asp Pro Leu Glu Leu Gln Glu
305 310 315 320

cta aac tcc ttg aag cag cag aaa gtc ata atc ctg gac ctg ttc agg 1129
Leu Asn Ser Leu Lys Gln Gln Lys Val Ile Ile Leu Asp Leu Phe Arg
325 330 335

act cgg aac att gcc acc ata acc gtg atg gct gtg atg ctg tgg atg 1177
Thr Arg Asn Ile Ala Thr Ile Thr Val Met Ala Val Met Leu Trp Met
340 345 350

cta acc tca gtg ggt tac ttt gct ctg tct ctc aat gtt cct aat tta 1225
Leu Thr Ser Val Gly Tyr Phe Ala Leu Ser Leu Asn Val Pro Asn Leu
355 360 365

cat gga gat gtc tac ctg aac tgc ttc ctc tct ggc ctg att gaa gtt 1273
His Gly Asp Val Tyr Leu Asn Cys Phe Leu Ser Gly Leu Ile Glu Val
370 375 380

cca gct tac ttc aca gcc tgg ctg cta ctg cga acc ctg cca cgg aga 1321
Pro Ala Tyr Phe Thr Ala Trp Leu Leu Leu Arg Thr Leu Pro Arg Arg

| | | | | |
|---|-----|-----|-----|------|
| 385 | 390 | 395 | 400 | |
| tat att ata gct ggg gtg cta ttc tgg gga gga ggt gtg ctt ctc ttg | | | | 1369 |
| Tyr Ile Ile Ala Gly Val Leu Phe Trp Gly Gly Gly Val Leu Leu Leu | | | | |
| | 405 | 410 | 415 | |
| atc caa gtg gta cct gaa gat tat aac ttt gtg tcc att gga ctg gtg | | | | 1417 |
| Ile Gln Val Val Pro Glu Asp Tyr Asn Phe Val Ser Ile Gly Leu Val | | | | |
| | 420 | 425 | 430 | |
| atg ctg ggg aaa ttt ggg atc acc tct gcc ttc tcc atg ttg tat gtc | | | | 1465 |
| Met Leu Gly Lys Phe Gly Ile Thr Ser Ala Phe Ser Met Leu Tyr Val | | | | |
| | 435 | 440 | 445 | |
| ttc act gcg gag ctc tac cca acc ctg gtc agg aac atg gct gtg ggc | | | | 1513 |
| Phe Thr Ala Glu Leu Tyr Pro Thr Leu Val Arg Asn Met Ala Val Gly | | | | |
| | 450 | 455 | 460 | |
| atc acc tcc atg gcc tct cgg gtg ggc agc atc att gcc ccc tat ttc | | | | 1561 |
| Ile Thr Ser Met Ala Ser Arg Val Gly Ser Ile Ile Ala Pro Tyr Phe | | | | |
| | 465 | 470 | 475 | 480 |
| gtt tac ctg ggc gcc tat aac aga ctc cta ccc tac atc ctc atg ggc | | | | 1609 |
| Val Tyr Leu Gly Ala Tyr Asn Arg Leu Leu Pro Tyr Ile Leu Met Gly | | | | |
| | 485 | 490 | 495 | |
| agt ctg act gtc ctc att gga atc atc acg ctt ttt ttc cct gaa agt | | | | 1657 |
| Ser Leu Thr Val Leu Ile Gly Ile Ile Thr Leu Phe Phe Pro Glu Ser | | | | |
| | 500 | 505 | 510 | |
| ttt gga gtg act cta cca gag aac ttg gag cag atg cag aaa gtg aga | | | | 1705 |
| Phe Gly Val Thr Leu Pro Glu Asn Leu Glu Gln Met Gln Lys Val Arg | | | | |
| | 515 | 520 | 525 | |
| ggg ttc aga tgt ggg aaa aaa tca aca gtc tca gtg gac aga gaa gaa | | | | 1753 |
| Gly Phe Arg Cys Gly Lys Lys Ser Thr Val Ser Val Asp Arg Glu Glu | | | | |
| | 530 | 535 | 540 | |
| agc ccc aag gtt cta ata act gca ttc taacgagggtt tccaaggcac | | | | 1800 |
| Ser Pro Lys Val Leu Ile Thr Ala Phe | | | | |
| | 545 | 550 | | |
| ttggcaaact gaaaagcaga tgtatacaat gagcagggtg tgatagagca agcctgcaat | | | | 1860 |

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<210> 24
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence
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<210> 25
<211> 24
<212> DNA
<213> Artificial Sequence
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<400> 25
acagaacaga aaagccctca gtca                                     24
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<220>

<223> Description of Artificial Sequence: Artificial Synthesized Primer Sequence

<400> 26

24

<213> Mus musculus

Met Gly Met Gln Thr Gly Phe Ser Phe Leu Gln Val Phe Ser Val Asn
180 185 190

Phe Glu Met Phe Thr Val Leu Phe Val Leu Val Gly Met Gly Gln Ile
 195 200 205
 Ser Asn Tyr Val Ala Ala Phe Val Leu Gly Thr Glu Ile Leu Ser Lys
 210 215 220
 Ser Ile Arg Ile Ile Phe Ala Thr Leu Gly Val Cys Ile Phe Tyr Ala
 225 230 235 240
 Phe Gly Phe Met Val Leu Pro Leu Phe Ala Tyr Phe Ile Arg Asp Trp
 245 250 255
 Arg Met Leu Leu Leu Ala Leu Thr Val Pro Gly Val Leu Cys Gly Ala
 260 265 270
 Leu Trp Trp Phe Ile Pro Glu Ser Pro Arg Trp Leu Ile Ser Gln Gly
 275 280 285
 Arg Ile Lys Glu Ala Glu Val Ile Ile Arg Lys Ala Ala Lys Ile Asn
 290 295 300
 Gly Ile Val Ala Pro Ser Thr Ile Phe Asp Pro Ser Glu Leu Gln Asp
 305 310 315 320
 Leu Asn Ser Thr Lys Pro Gln Leu His His Ile Tyr Asp Leu Ile Arg
 325 330 335
 Thr Arg Asn Ile Arg Val Ile Thr Ile Met Ser Ile Ile Leu Trp Leu
 340 345 350
 Thr Ile Ser Val Gly Tyr Phe Gly Leu Ser Leu Asp Thr Pro Asn Leu
 355 360 365
 His Gly Asp Ile Tyr Val Asn Cys Phe Leu Leu Ala Ala Val Glu Val
 370 375 380
 Pro Ala Tyr Val Leu Ala Trp Leu Leu Leu Gln Tyr Leu Pro Arg Arg
 385 390 395 400
 Tyr Ser Ile Ser Ala Ala Leu Phe Leu Gly Gly Ser Val Leu Leu Phe
 405 410 415

Met Gln Leu Val Pro Ser Glu Leu Phe Tyr Leu Ser Thr Ala Leu Val
 420 425 430

Met Val Gly Lys Phe Gly Ile Thr Ser Ala Tyr Ser Met Val Tyr Val
 435 440 445

Tyr Thr Ala Glu Leu Tyr Pro Thr Val Val Arg Asn Met Gly Val Gly
 450 455 460

Val Ser Ser Thr Ala Ser Arg Leu Gly Ser Ile Leu Ser Pro Tyr Phe
 465 470 475 480

Val Tyr Leu Gly Ala Tyr Asp Arg Phe Leu Pro Tyr Ile Leu Met Gly
 485 490 495

Ser Leu Thr Ile Leu Thr Ala Ile Leu Thr Leu Phe Phe Pro Glu Ser
 500 505 510

Phe Gly Val Pro Leu Pro Asp Thr Ile Asp Gln Met Leu Arg Val Lys
 515 520 525

Gly Ile Lys Gln Trp Gln Ile Gln Ser Gln Thr Arg Met Gln Lys Asp
 530 535 540

Gly Glu Glu Ser Pro Thr Val Leu Lys Ser Thr Ala Phe
 545 550 555

<210> 28

<211> 1888

<212> DNA

<213> Mus musculus

<220>

<221> CDS

<222> (60)..(1730)

<400> 28

ctcccgcgcc acggtgtccc cttattccca tacgggcgct gtgggaggct gaggacggc 59

atg cgg gac tac gac gag gtg acc gcc ttc cta ggc gag tgg ggg ccc 107
 Met Arg Asp Tyr Asp Glu Val Thr Ala Phe Leu Gly Glu Trp Gly Pro
 1 5 10 15

| | |
|---|-----|
| ttc cag cgc ctc atc ttc ttc ctg ctc agc gcc agc atc atc ccc aat | 155 |
| Phe Gln Arg Leu Ile Phe Phe Leu Leu Ser Ala Ser Ile Ile Pro Asn | |
| 20 25 30 | |
| ggc ttc aat ggt atg tcc atc gtg ttc ctg gcg ggg acc ccg gag cac | 203 |
| Gly Phe Asn Gly Met Ser Ile Val Phe Leu Ala Gly Thr Pro Glu His | |
| 35 40 45 | |
| cgt tgc ctt gtg cct cac acc gtg aac ctg agc agc gcg tgg cgc aac | 251 |
| Arg Cys Leu Val Pro His Thr Val Asn Leu Ser Ser Ala Trp Arg Asn | |
| 50 55 60 | |
| cac agt atc ccg ttg gag acg aag gac gga cga cag gtg cct cag aaa | 299 |
| His Ser Ile Pro Leu Glu Thr Lys Asp Gly Arg Gln Val Pro Gln Lys | |
| 65 70 75 80 | |
| tgc cgc cgc tac cga ctg gcc acc atc gcc aac ttc tct gag cta ggg | 347 |
| Cys Arg Arg Tyr Arg Leu Ala Thr Ile Ala Asn Phe Ser Glu Leu Gly | |
| 85 90 95 | |
| ctg gag ccg ggg cgg gac gtg gac ctg gag cag ctg gag cag gag agc | 395 |
| Leu Glu Pro Gly Arg Asp Val Asp Leu Glu Gln Leu Glu Gln Glu Ser | |
| 100 105 110 | |
| tgc ctg gat ggc tgg gag tac gac aag gac gtc ttc ctg tcc acc atc | 443 |
| Cys Leu Asp Gly Trp Glu Tyr Asp Lys Asp Val Phe Leu Ser Thr Ile | |
| 115 120 125 | |
| gtg aca gag tgg gac ctg gtg tgt aag gat gac tgg aaa gcc cca ctc | 491 |
| Val Thr Glu Trp Asp Leu Val Cys Lys Asp Asp Trp Lys Ala Pro Leu | |
| 130 135 140 | |
| acc acc tcc ttg ttt ttc gtg ggt gtg ctg atg gcc tcc ttc att tca | 539 |
| Thr Thr Ser Leu Phe Phe Val Gly Val Leu Met Gly Ser Phe Ile Ser | |
| 145 150 155 160 | |
| gga cag ctc tca gac agg ttt ggt cgc aag aat gtg ctg ttt ttg acc | 587 |
| Gly Gln Leu Ser Asp Arg Phe Gly Arg Lys Asn Val Leu Phe Leu Thr | |
| 165 170 175 | |
| atg gcc atg cag act gcc ttc agc ttc ctg cag gtc ttc tct gtg aac | 635 |
| Met Gly Met Gln Thr Gly Phe Ser Phe Leu Gln Val Phe Ser Val Asn | |
| 180 185 190 | |

tcc aac tac gtg gca gca ttt gtc ctg gga aca gaa att ctt tcc aag 731
Ser Asn Tyr Val Ala Ala Phe Val Leu Gly Thr Glu Ile Leu Ser Lys
210 215 220

ttt ggc ttc atg gtg ctg cca ctg ttt gca tac ttc atc aga gac tgg 827
Phe Gly Phe Met Val Leu Pro Leu Phe Ala Tyr Phe Ile Arg Asp Trp
245 250 255

agg atg ctg ctg ctg gcg ctc act gtg cca ggg gtg cta tgt ggg gct 875
Arg Met Leu Leu Leu Ala Leu Thr Val Pro Gly Val Leu Cys Gly Ala
260 265 270

ctc tgg tgg ttc atc cct gag tcc cca cga tgg ctc atc tct caa ggc 923
Leu Trp Trp Phe Ile Pro Glu Ser Pro Arg Trp Leu Ile Ser Gln Gly
275 280 285

cga att aaa gag gca gag gtg atc atc cgc aaa gct gcc aaa atc aat 971
Arg Ile Lys Glu Ala Glu Val Ile Ile Arg Lys Ala Ala Lys Ile Asn
290 295 300

ggg att gtt gca cct tcc act atc ttc gat cca agt gag tta caa gac 1019
Gly Ile Val Ala Pro Ser Thr Ile Phe Asp Pro Ser Glu Leu Gln Asp
305 310 315 320

tta aat tct acg aag cct cag ttg cac cac att tat gat ctg atc cga 1067
Leu Asn Ser Thr Lys Pro Gln Leu His His Ile Tyr Asp Leu Ile Arg
325 330 335

aca cgg aat atc agg gtc atc acc atc atg tct ata atc ctg tgg ctg 1115
Thr Arg Asn Ile Arg Val Ile Thr Ile Met Ser Ile Ile Leu Trp Leu
340 345 350

acc ata tca gtg ggc tat ttt gga cta tct ctt gac act cct aac ttg 1163
Thr Ile Ser Val Gly Tyr Phe Gly Leu Ser Leu Asp Thr Pro Asn Leu

| | | | |
|---|-----|-----|------|
| 355 | 360 | 365 | |
| cat ggg gac atc tat gtg aac tgc ttc cta ctg gcg gct gtt gaa gtc | | | 1211 |
| His Gly Asp Ile Tyr Val Asn Cys Phe Leu Leu Ala Ala Val Glu Val | | | |
| 370 | 375 | 380 | |
| cca gcc tat gtg ctg gcc tgg ctg ttg ttg cag tac ttg ccc cgg cga | | | 1259 |
| Pro Ala Tyr Val Leu Ala Trp Leu Leu Leu Gln Tyr Leu Pro Arg Arg | | | |
| 385 | 390 | 395 | 400 |
| tat tct atc tgc gct gcc ctt ttc ctg ggt ggc agt gtc ctt ctc ttc | | | 1307 |
| Tyr Ser Ile Ser Ala Ala Leu Phe Leu Gly Gly Ser Val Leu Leu Phe | | | |
| 405 | 410 | 415 | |
| atg cag ctg gtg cct tca gaa ttg ttt tac ttg tcc act gcc ctg gtg | | | 1355 |
| Met Gln Leu Val Pro Ser Glu Leu Phe Tyr Leu Ser Thr Ala Leu Val | | | |
| 420 | 425 | 430 | |
| atg gtg ggg aag ttt gga atc acc tct gcc tac tcc atg gtc tat gtg | | | 1403 |
| Met Val Gly Lys Phe Gly Ile Thr Ser Ala Tyr Ser Met Val Tyr Val | | | |
| 435 | 440 | 445 | |
| tac aca gct gag ctg tac ccc act gtg gtc aga aac atg ggt gtg ggg | | | 1451 |
| Tyr Thr Ala Glu Leu Tyr Pro Thr Val Val Arg Asn Met Gly Val Gly | | | |
| 450 | 455 | 460 | |
| gtc agc tcc aca gca tcc cgc ctt ggc agc atc ctg tct ccc tac ttt | | | 1499 |
| Val Ser Ser Thr Ala Ser Arg Leu Gly Ser Ile Leu Ser Pro Tyr Phe | | | |
| 465 | 470 | 475 | 480 |
| gtt tac cta ggt gcc tat gat cgc ttc ctg cct tat att ctc atg gga | | | 1547 |
| Val Tyr Leu Gly Ala Tyr Asp Arg Phe Leu Pro Tyr Ile Leu Met Gly | | | |
| 485 | 490 | 495 | |
| agt ctg acc atc ctg aca gct atc ctc acc ttg ttc ttc cct gag agc | | | 1595 |
| Ser Leu Thr Ile Leu Thr Ala Ile Leu Thr Leu Phe Phe Pro Glu Ser | | | |
| 500 | 505 | 510 | |
| ttt ggt gtc cct ctc cca gat acc att gac cag atg cta agg gtc aaa | | | 1643 |
| Phe Gly Val Pro Leu Pro Asp Thr Ile Asp Gln Met Leu Arg Val Lys | | | |
| 515 | 520 | 525 | |
| gga ata aaa cag tgg caa atc caa agc cag aca aga atg caa aaa gat | | | 1691 |

tggaagtca ccttcctcta gggacacc 1888

[illegible][illegible][illegible][illegible][illegible]

| | 1970 | 1971 | 1972 | 1973 | 1974 | 1975 | 1976 | 1977 | 1978 | 1979 | 1980 | 1981 | 1982 | 1983 | 1984 | 1985 | 1986 | 1987 | 1988 | 1989 | 1990 | 1991 | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 | 2021 | 2022 | 2023 | 2024 | 2025 | 2026 | 2027 | 2028 | 2029 | 2030 | 2031 | 2032 | 2033 | 2034 | 2035 | 2036 | 2037 | 2038 | 2039 | 2040 | 2041 | 2042 | 2043 | 2044 | 2045 | 2046 | 2047 | 2048 | 2049 | 2050 | 2051 | 2052 | 2053 | 2054 | 2055 | 2056 | 2057 | 2058 | 2059 | 2060 | 2061 | 2062 | 2063 | 2064 | 2065 | 2066 | 2067 | 2068 | 2069 | 2070 | 2071 | 2072 | 2073 | 2074 | 2075 | 2076 | 2077 | 2078 | 2079 | 2080 | 2081 | 2082 | 2083 | 2084 | 2085 | 2086 | 2087 | 2088 | 2089 | 2090 | 2091 | 2092 | 2093 | 2094 | 2095 | 2096 | 2097 | 2098 | 2099 | 2100 | 2101 | 2102 | 2103 | 2104 | 2105 | 2106 | 2107 | 2108 | 2109 | 2110 | 2111 | 2112 | 2113 | 2114 | 2115 | 2116 | 2117 | 2118 | 2119 | 2120 | 2121 | 2122 | 2123 | 2124 | 2125 | 2126 | 2127 | 2128 | 2129 | 2130 | 2131 | 2132 | 2133 | 2134 | 2135 | 2136 | 2137 | 2138 | 2139 | 2140 | 2141 | 2142 | 2143 | 2144 | 2145 | 2146 | 2147 | 2148 | 2149 | 2150 | 2151 | 2152 | 2153 | 2154 | 2155 | 2156 | 2157 | 2158 | 2159 | 2160 | 2161 | 2162 | 2163 | 2164 | 2165 | 2166 | 2167 | 2168 | 2169 | 2170 | 2171 | 2172 | 2173 | 2174 | 2175 | 2176 | 2177 | 2178 | 2179 | 2180 | 2181 | 2182 | 2183 | 2184 | 2185 | 2186 | 2187 | 2188 | 2189 | 2190 | 2191 | 2192 | 2193 | 2194 | 2195 | 2196 | 2197 | 2198 | 2199 | 2200 | 2201 | 2202 | 2203 | 2204 | 2205 | 2206 | 2207 | 2208 | 2209 | 2210 | 2211 | 2212 | 2213 | 2214 | 2215 | 2216 | 2217 | 2218 | 2219 | 2220 | 2221 | 2222 | 2223 | 2224 | 2225 | 2226 | 2227 | 2228 | 2229 | 2230 | 2231 | 2232 | 2233 | 2234 | 2235 | 2236 | 2237 | 2238 | 2239 | 2240 | 2241 | 2242 | 2243 | 2244 | 2245 | 2246 | 2247 | 2248 | 2249 | 2250 | 2251 | 2252 | 2253 | 2254 | 2255 | 2256 | 2257 | 2258 | 2259 | 2260 | 2261 | 2262 | 2263 | 2264 | 2265 | 2266 | 2267 | 2268 | 2269 | 2270 | 2271 | 2272 | 2273 | 2274 | 2275 | 2276 | 2277 | 2278 | 2279 | 2280 | 2281 | 2282 | 2283 | 2284 | 2285 | 2286 | 2287 | 2288 | 2289 | 2290 | 2291 | 2292 | 2293 | 2294 | 2295 | 2296 | 2297 | 2298 | 2299 | 2300 | 2301 | 2302 | 2303 | 2304 | 2305 | 2306 | 2307 | 2308 | 2309 | 2310 | 2311 | 2312 | 2313 | 2314 | 2315 | 2316 | 2317 | 2318 | 2319 | 2320 | 2321 | 2322 | 2323 | 2324 | 2325 | 2326 | 2327 | 2328 | 2329 | 2330 | 2331 | 2332 | 2333 | 2334 | 2335 | 2336 | 2337 | 2338 | 2339 | 2340 | 2341 | 2342 | 2343 | 2344 | 2345 | 2346 | 2347 | 2348 | 2349 | 2350 | 2351 | 2352 | 2353 | 2354 | 2355 | 2356 | 2357 | 2358 | 2359 | 2360 | 2361 | 2362 | 2363 | 2364 | 2365 | 2366 | 2367 | 2368 | 2369 | 2370 | 2371 | 2372 | 2373 | 2374 | 2375 | 2376 | 2377 | 2378 | 2379 | 2380 | 2381 | 2382 | 2383 | 2384 | 2385 | 2386 | 2387 | 2388 | 2389 | 2390 | 2391 | 2392 | 2393 | 2394 | 2395 | 2396 | 2397 | 2398 | 2399 | 2400 | 2401 | 2402 | 2403 | 2404 | 2405 | 2406 | 2407 | 2408 | 2409 | 2410 | 2411 | 2412 | 2413 | 2414 | 2415 | 2416 | 2417 | 2418 | 2419 | 2420 | 2421 | 2422 | 2 |
|--|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|---|
|--|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|---|

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